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PREFACE

The Editorial Committee has continued its policy of selecting subjects for review in areas where significant progress has been made over the past several years. These subjects are reviewed in depth by competent investigators in the particular fields chosen. Originally, an attempt was made to cover the various subspecialties of medicine. However, it is becoming apparent that there is considerable crossover between these subspecialties. For instance, in this volume will be found linkages between psychiatry and genetics, psychiatry and pharmacology, pharmacology and genetics, surgery and gastroenterology, surgery and cardiology, and many others. It is important, therefore, that the scientific investigator in the medical sciences and the practicing physician be kept abreast of advances in fields other than those in which he is especially interested. We have attempted to satisfy these needs with the carefully selected reviews contained in this volume. We have been fortunate in having experts in each subject write the reviews. We thank our contributors for the competent job they have done. The reviews are well written and can be read with pleasure. Also, we are thankful to our ever patient, hardworking Assistant Editor, Miss Beryl Daniel, whose careful work has kept errors from creeping into the text, and has thus materially lessened the work of the Editor and Associate Editor. We also thank our printers, the George Banta Company, for again doing an excellent printing job.

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REPRINTS

The conspicuous number (7026 to 7054) aligned in the margin with the title of each chapter in this volume is a key for use in the ordering of reprints.

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ABLATION THERAPY FOR HORMONE-DEPENDENT TUMORS

7026

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INTRODUCTION

Although excision of endocrine glands to control human cancer was first introduced in 1896 by Beatson (1) who performed bilateral oophorectomy in two women with breast cancer, the modern era of endocrine surgery for human cancer was born in 1940, when Huggins (2) first reported the dramatic effect of orchiectomy in men with cancer of the prostate. This endocrine intervention is based on the concept of "hormone-dependence," a concept originated from the classic experiments of Huggins in his study of the effects of hormones on prostatic secretion (3). This concept evolved from the recognition of the relationship between hormonal target tissues and their neoplastic counterparts and suggests that neoplasms of target tissues retain sufficient characteristics of the normal cells from which they arose that the tumor cells function very much like their tissues of origin. When the original cells are dependent on hormonal support for metabolic activity, the cells of a neoplasm derived from the original cells can be similarly dependent, and they involute or atrophy when endocrine support is withdrawn.

The discovery of hormone dependence thus represents a break-away from the long accepted notion of "autonomy" of a malignant neoplasm. Rous, discussing the implications of the discovery, wrote that "the significance of this discovery far transcends its practical implications; for it means that thought and endeavor in cancer research have been misdirected in consequence of belief that tumor cells are anarchic" (4). The 1966 Nobel Prize for Medicine or Physiology was awarded jointly to Charles Huggins and Peyton Rous for their contributions into cancer research.

The growth of interest in the control of advanced cancers by endocrinological methods has led to widespread use of endocrine-ablative procedures, particularly in cancers of the breast and prostate, in the last two decades. Although control of cancer of the prostate by orchiectomy is well established, adrenalectomy or hypophysectomy has not been proved to induce prostatic tumor regression that cannot also be obtained by other simple modalities of therapy. In contrast, adrenalectomy or hypophysectomy has been shown to give men with advanced cancer of the breast benefit that no other method of treatment can yet achieve. Recent years have also witnessed a surge of attempts to determine the mechanism of regression of breast cancer in women following endocrine ablation, and thus to find methods for distinguishing "endocrine-dependent" from "endocrine-independent" cancers. Of particular significance should be definition of the hormonal environment in which the neoplastic disease evolves.

The purposes of this review are to examine the role of endocrine ablations in the treatment of mammary cancer and to discuss various laboratory studies concerning the mechanism of endocrine factors influencing tumor growth. Recent advances in the study of hormone receptors and of the metabolism of steroid hormones by breast cancer tissues are of particular significance in our eventual understanding of the role of hormones in the pathogenesis of cancer. No attempt is made to provide complete coverage of the literature, nor is this thought to be necessary.

CASTRATION

Historical considerations.—Sir George Beatson was the first to prove that breast cancer could be induced to regress by excision of the ovaries. Some of the considerations that led Beatson to perform oophorectomy follow his observations on a farm: (*a*) the secretion of milk is not affected by sections of the sympathetic or spinal nerves; (*b*) "the process of lactation is almost identical, up to a certain point, with what takes place in the cancerous mamma"; and (*c*) removal of the ovaries of lactating cows prolongs milk production indefinitely. These facts pointed to one organ's "holding control over another and separate organ in the absence of nervous control." The question arose in Beatson's mind: "Is cancer of the mamma due to some ovarian irritation?" Beatson performed oophorectomy in two women with advanced mammary cancer in 1896; in both cases, regression of cancer was observed (5).

Despite Beatson's successful pioneering study, he introduced oophorectomy at a time when there was no concept of endocrinology, and the operation had largely fallen into disfavor by 1905. The isolation of estrone in 1929 independently by Doisy (6) in the United States and by Butenandt (7) in Germany, led to Lacassagne's classic experiment (8) in which he induced mammary tumors in male mice by giving them injections of estrone. This experiment established a causal relationship between estrogenic hormones and mammary tumorigenesis. On the basis of Huggins' report of the success of orchiectomy in men with advanced cancer of the prostate, the efficacy of castration, although known to be inconstant was re-evaluated. Numerous reports of the results of bilateral oophorectomy in women with metastatic breast cancer have appeared in the medical literature since 1945.

Clinical results of "therapeutic" castration.—"Therapeutic" castration is defined as castration performed as a palliative measure in women with locally advanced (surgically inoperable) breast cancer and in those with recurrent or metastatic lesions.

Bilateral oophorectomy has proved to be an effective palliative therapy in approximately a third of premenopausal women with disseminated cancer of the breast. The data from several large series indicate that 20–45 percent of premenopausal patients with advanced breast cancer obtained beneficial ef-

TABLE 1. OBJECTIVE REMISSION AFTER SURGICAL CASTRATION IN FOUR PUBLISHED SERIES

Authors	Total no. of cases	No. of remission	Percentage of remission
Pearson et al (9)	75 Premenopausal	33	44
	21 Postmenopausal	2	10
Block et al (10)	26 Premenopausal	9	35
	20 Postmenopausal	4	20
Dao (11)	158 Premenopausal	64	40
	49 Menopausal[a]	8	16
Taylor (12)	381	113	30

[a] Menopausal is defined as the period between cessation of menstruation to one year postmenopausal.

fects from surgical castration (Table 1). In hormone-dependent breast cancer, metastases will respond to castration uniformly, irrespective of their site of involvement. Remission consists not only of symptomatic relief of disabling symptoms due to bone and lung metastases, but also objective evidence of tumor regression, including (*a*) decrease or disappearance of grossly or radiographically discernible or palpable tumors; (*b*) decrease or disappearance of cancerous exudates; (*c*) sclerosis of previously osteolytic or disappearance of previously osteoblastic bone metastases; (*d*) recovery from paralysis in patients with CNS or spinal cord lesions; and (*e*) decrease in the size of metastases of abdominal organs, such as the liver. Response to castration in patients with liver metastases depends largely on the extent to which the liver is involved (13).

The beneficial effects of castration are essentially confined to premenopausal women. Although castration may occasionally be of value a few years after menopause, the operation is not justified unless there is evidence of residual ovarian activity. In our series of 202 consecutive cases, we observed that the incidence of remission was significantly smaller when oophorectomy was performed in menopausal patients (last menstrual period, 2–12 months). No objective regression was observed if castration was done in post-menopausal women. Figure 1 illustrates the influence of menstrual status on the incidence of remission.

Is there a role for prophylactic castration?—The poor results of mastectomy in young women with breast cancer and the apparent benefits of castration in some women with metastatic breast cancer led to investigation of performing castration at the time of radical mastectomy to achieve improvement in the cure rate. Since the early reports of Taylor (14) and Horsley (15), there has been widespread disagreement as to whether prophylactic castration increases the cure rate in premenopausal patients undergoing radical mastectomy for operable breast cancer. In almost all of the earlier reports, the results came from retrospective studies lacking control series of patients. It has been thought that the conclusions were inadequate and often opinionated.

The National Surgical Adjuvant Breast Project carried out a randomized clinical trial to determine the worth of oophorectomy as an adjuvant to radical mastectomy for the treatment of operable carcinoma of the breast in premenopausal women. The data obtained show no difference at any time, in either the recurrence rate or the survival rate, between patients undergoing prophylactic castration and those in the control group. Where patients were classified according to their nodal status—i.e., positive or negative (Table 2)—no advantage was obtained for patients after oophrectomy. On the basis of these findings, it has been concluded that prophylactic castration is not justified in the treatment of operable breast carcinoma (16).

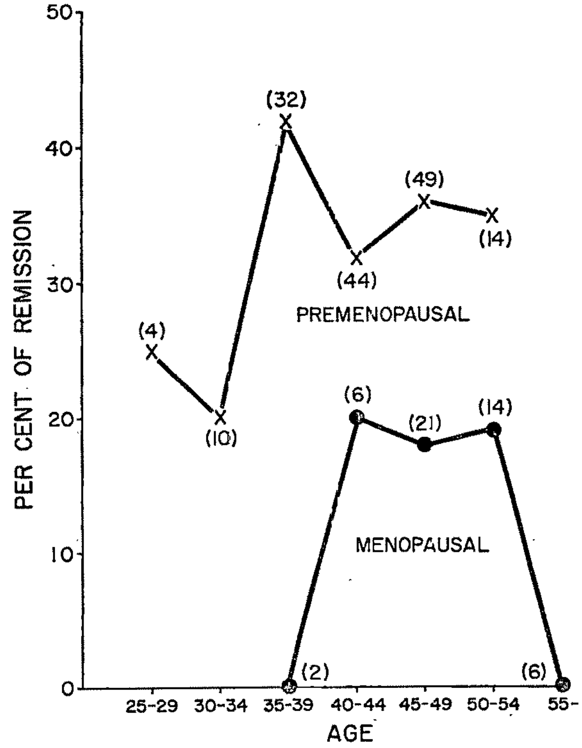

Fig. 1. Age and Menopausal studies in relation to response to castration in women with mestastatic cancer of the breast. Numbers in parentheses are number of patients.

TABLE 2. Recurrence and Survival Rate at Three, Four, and Five Years After "Prophylactic" Castration

Months after Operation	Recurrence rate				Survival rate			
	Negative nodes		Positive nodes		Negative nodes		Positive nodes	
	Control	Castration	Control	Castration	Control	Castration	Control	Castration
36	16%	21%	59%	55%	88%	87%	64%	59%
48	20%	22%	61%	63%	83%	80%	59%	51%
60	33%	26%	74%	76%	72%	77%	43%	39%

Surgical versus roentgen castration.—Surgical castration essentially guarantees the complete eradication of ovarian function. The effect is rapid, and is often desirable in patients suffering from disabling and acute symptoms such as hypercalcemia and crippling bone pain, respiratory difficulties in those with pleuropulmonary metastasis, and neurological symptoms in many with brain metastasis. In contrast, evidence of ovarian suppression following ovarian irradiation does not appear before the end of a period of six to eight weeks. The beneficial effects of castration thus do not develop rapidly in patients receiving ovarian irradiation.

Controversy has arisen concerning the dose of radiation required for achieving complete suppression of ovarian activity. Dosage schedules ranging from 600 to 1500 r delivered either as a single dose (600 r) or in divided doses within 10 days have been employed. Recovery of ovarian function may occur even after an adequate "sterilizing" dose has been used.

ADRENALECTOMY

The important considerations that led to association between the adrenal gland and human cancer are these: First, the adrenal cortex is known to be involved as an etiologic agent in the genesis of certain mammary tumors of mice. When the gonads of certain stocks of mice are removed at an early age, adrenal tumors occur (17). Shimkin & Wyman found that adrenalectomy reduced the incidence of spontaneous mammary cancer in C_3H mice fully as much as did oophorectomy (18). Second, there is pathological and laboratory evidence for the elaboration of hormones from the adrenal cortex in human subjects having either adrenal hyperfunction or adrenal tumors. Increased growth of secondary sexual features, as well as fatal metabolic disturbances, can result from an excess of steroid hormone production in patients with adrenocortical hyperplasia or tumors (19). Third, orchiectomy in human males is usually followed by an increase in urinary 17-ketosteroids. Bilateral adrenalectomy reduces the high values to only trace amounts (20). Fourth, oophorectomy in human females induces an abrupt drop in the urinary estrogen level, but this initial drop is often followed by a gradual increase, frequently to amounts above the precastration levels (21). These findings are evidence indicating that another locus besides the ovaries, probably the adrenal cortex, produces C_{19} and C_{18} steroids.

Although bilateral total adrenalectomy was first performed as long ago as 1945 (22), hormonal maintenance was not satisfactory at that time, because of an inadequate supply of II-oxygenated steroids. Bilateral adrenalectomy is no longer a perilous operation, however, now that cortisone has been available in abundance since 1951 (23).

Substitution therapy after total adrenalectomy.—From the practical standpoint, adrenal insufficiency must be forestalled. It is a safe rule in any proposed extirpation of the adrenals to treat the patient in advance as if adrenal insufficiency is certain to develop after surgery, and to withdraw the essential hormones slowly during the postoperative period. The substitution therapy originally devised at the University of Chicago (24) has permitted us to do many hundreds of adrenalectomies without postoperative adrenal insufficiency. It essentially involves the use of rather large amounts of parenterally administered cortisone acetate the day preceding the operation and the day of the operation. On subsequent days, the amount of cortisone is gradually reduced. Frequently the patient can be placed on oral cortisone acetate by the third or fourth postoperative day. Deoxycorticosterone acetate (DOCA) is rarely needed; the amount given depends upon how rapidly the blood pressure stabilizes at a satisfactory level. Usually by the sixth postoperative day, the patient will be on her future oral maintenance dosage of cortisone acetate. Supplementary sodium chloride, which was used in our replacement therapy, is no longer given routinely.

With increased experience in adrenal surgery for cancer and in the clinical management of adrenalectomized patients, the original schedule for corticosteroid replacement therapy, particularly for preoperative management, has been modified. The necessity for large doses of cortisone the day before and the day of the operation has been greatly reduced. We have found that it is no longer necessary for preoperative preparation in patients undergoing adrenalectomy.

The criterion for adequate hormonal substitution in adrenalectomized patients is the prevention of any sign or symptom of adrenal insufficiency. Probably the best indicator is the sense of well-being of the patient. When maintained adequately, adrenalectomized patients have a healthy appearance, are not incapacitated, and are able to engage in most of their usual activities.

Early recognition of the symptoms and signs of adrenal insufficiency is imperative if adrenal crisis is to be prevented. Acute adrenal crisis is almost always ushered in with some concomitant disease. Severe illness, such as acute infectious diseases, acute abdominal conditions (mostly surgical, such as cholecystitis, appendicitis, intestinal obstruction, ruptured viscus, or trauma), and many other stress-producing circumstances, will induce adrenal crisis which, unless vigorously and promptly treated, means certain

TABLE 3. Objective Remission After Adrenalectomy in Seven Published Series

Authors	Total no. of cases	No. of remission	Percentage of remission
Dao & Huggins (26)	100	39	39
Cade (27)	136	54	40
Pyrah (28)	75	39	52
Fracchia et al (29)	500	178	36
Hellstrom & Franksson (30)	233	100	42
Delarue et al (31)	133	89	67
Dao et al (32)	207	83	40

death to the patient. When adrenalectomized patients undergo major surgical procedures, they must be prepared as if they were to have adrenalectomy. In minor surgery, cortisone doses should be slightly increased before and immediately after the procedure.

Clinical results of adrenalectomy.—The beneficial effects of bilateral adrenalectomy for women with metastatic breast cancer are now well established. Table 3 summarizes the results of the larger series of adrenalectomies published to date. Remission rates reported in the papers were based on the observation of demonstrable evidence of tumor regression following adrenalectomy. Objective response ranged between 39 and 50 percent, suggesting that the criteria used in the various investigations are quite uniform. The objective criteria for tumor regression have been discussed earlier in this paper.

Adrenalectomy is almost equally effective in patients with soft-tissue, osseous, and visceral metastases. Metastases in the liver is not a contraindication for adrenalectomy. Response to adrenalectomy in women with hepatic metastases depends largely on the extent to which the liver is involved. In our study we find that adrenalectomy has little value if more than 30 percent of the liver is replaced with metastases (25).

Cerebral metastasis without clinical manifestations of seizures is not a contraindication for adrenalectomy. Even in women with hemiplegia, we have observed remarkable objective regression and complete restoration of motor function lasting for many months after adrenalectomy in four patients. Cerebellar metastases with disabling clinical symptoms often respond favorably to adrenalectomy.

Early (primary) versus late (secondary) adrenalectomy.—In view of the noteworthy palliation of advanced cancer by adrenalectomy, it is obviously desirable to consider the possibility of increasing benefits if the procedure is done at the outset when metastases first become demonstrable (primary adrenalectomy). A controlled randomized comparative study was carried out in our clinic to determine whether there is a difference between primary and secondary adrenalectomy (adrenalectomy after adequate trial of hormonal therapy). The detailed results have been published earlier (33). The data can be briefly summarized as follows:

First, the objective regression rate is considerably better for primary adrenalectomy (46 percent) than for secondary adrenalectomy (30 percent).

Secondary, adrenalectomy is therapeutically much more effective than hormone therapy.

Third, neither response nor lack of response to previous hormonal therapy can be correlated with subsequent response to adrenalectomy. The study showed that objective remission following adrenalectomy occurred in 30 percent of hormone responders and 37 percent of hormone nonresponders.

Fourth, previous response to surgical castration is a definitely favorable indication for subsequent response to adrenalectomy. Our study revealed that at least 50 percent of the castration responders would respond again to adrenalectomy.

Fifth, survival is significantly longer for patients responding to adrenalectomy than for patients who fail to respond.

Is castration necessary in postmenopausal women undergoing adrenalectomy?—In our earlier experience, adrenalectomy was always done in combination with oophorectomy on the premise that postmenopausal women continued to have ovarian activity. There has been no conclusive evidence to show that the ovaries in late postmenopausal women continue to secrete significant amounts of estrogenic hormones. Although there have been reports suggesting a better remission rate for patients having combined oophorectomy and adrenalectomy, none of the data was convincing. Our clinical experience in the last ten years has shown no significant changes in remission and survival rates in postmenopausal patients who underwent adrenalectomy without simultaneous oophorectomy. In the last 750 consecutive cases of adrenalectomies (without oophorectomy), we have observed a tumor regression rate of 45 percent, a result that is in good agreement with those appearing in early reports of combined oophorectomy and adrenalectomy. It is conceivable that some late menopausal women still have ovaries producing significant amounts of estrogenic hormones. Extended studies are being carried out in our laboratory. At present, the only indication for combined oophorectomy and adrenalectomy in postmenopausal patients is recent menopause (cessation of menstruation within one year before surgery).

Metabolic effects of adrenalectomy.—The adrenalectomized patients can be maintained in a state of good health for indefinite periods with replacement cortisone therapy.

The clinical and laboratory findings of hypoglycemia and flattened oral glucose tolerance curves frequently seen in patients with Addison's disease maintained on salt and DOCA, were not observed in adrenalectomized patients maintained adequately on cortisone acetate. There have been hypoglycemic episodes after intravenous glucose tolerance tests (34).

Symptoms of hypoglycemia fail to develop even after a long period of starvation. The intravenous insulin tolerance test was essentially the same in pre- and postoperative periods. Patients with diabetes mellitus required the same insulin dosage after adrenalectomy while on a maintenance dose of 50 mg of cortisone acetate orally.

Patients with Addison's disease fail to demonstrate adequate water diuresis when they are given water loads (Robinson-Kepler-Power water test). Adrenalectomized patients on cortisone maintenance show adequate diuresis during water loading test. These patients also do not have altered sodium metabolism if adequate salt intake is maintanied. They can maintain sodium balance, and measurements of serum sodium and total exchangeable body sodium are usually within the normal limits. Sodium restrictions in these patients, however, will induce renal salt wastage and thus the development of a low sodium syndrome (35). This inability to conserve sodium is believed to be due to the absence of aldosterone. In about 10 percent of our adrenalectomized patients, active sodium-retaining steroid is required to insure renal conservation of sodium. We use a long-acting compound, deoxycorticosterone-trimethylacetate, 25 mg once a month to offer some protection against sodium depletion.

HYPOPHYSECTOMY

That the pituitary gland has an important role in the genesis of mammary cancer and in the maintenance of its growth has been convincingly demonstrated in experimental animals (36). Growth hormone has been shown to have a stimulating effect on tumor growth in experimental animals and in man. It is only recently, however, that substantial evidence has been obtained to show that prolactin is likely to be the most important pituitary hormone in the control of growth of cancer of the breast (37, 38).

The first application of hypophysectomy for malignant disease was reported in 1952 by three groups: Scott & Walker for cancer of the prostate (39), Shimkin et al for malignant melanoma (40), and Perrault et al (41) for cancer of the breast. The beneficial effects of hypophysectomy on human breast cancer were actually first demonstrated by Luft & Olivecrona (42) in Sweden, and by Pearson & Ray (43) in the United States.

Clinical results of hypophysectomy.—The benefits from surgical hypophysectomy have been similar to those following adrenalectomy. Metastatic lesions of all sites have been shown to regress after hypophysectomy in about 40 percent of patients. The remission rates in several large and comparable series of transcranial hypophysectomy are shown in Table 4. The duration of remission following hypophysectomy is similar to that after adrenalectomy. Survival is significantly longer for patients responding to hypophysectomy than for those who fail to respond.

Attempts at comparing the therapeutic effects of adrenalectomy and hypophysectomy have been made by several groups, and the results fail to demonstrate any significant difference between these two ablative procedures. Several investigators consider that the practical advantages of adrenalectomy, including (*a*) less morbidity, (*b*) simpler replacement therapy, and (*c*) the fact that the procedure can be performed by most trained surgeons, are important considerations in favor of adrenalectomy.

Metabolic effects of hypophysectomy.—Total removal of the pituitary gland in man causes suppression of the functions of the gonads, the thyroid, and the adrenal cortex. Hypophysectomy induces a marked suppression of thyroid function. Li *et al* (48) reported clinical manifestations of hypothyroidism in man usually within four to six weeks after hypophysectomy. Serum protein-bound iodine and uptake of I^{131} by the thyroid fall rapidly to myxedema levels within a couple of weeks after surgery. Serum cholesterol levels usually are moderately elevated.

Menstruation ceases when hypophysectomy is performed before menopause. Pituitary gonadotropin excretion usually becomes unmeasurable within two to three weeks after the operation. Clinical manifestations of menopause ensue. Ovaries in females and testes in males atrophy and become greatly reduced in size.

After hypophysectomy, cortisone replacement therapy is necessary. Upon withdrawal of cortisone from hypophysectomized patients, blood and urinary 17-OH corticosteroids fall to very low levels, and signs and symptoms of adrenal crisis appear shortly afterwards. McLean et al (49) reported that aldosterone secretion by the adrenal cortex did not seem to be significantly affected by removal of pituitary gland. It was observed that renal concentration of sodium occurs in hypophysectomized patients during sodium restriction, and urinary excretion of sodium-retaining corticosteroid hormones was greatly increased. These findings suggest that aldosterone secretion by the adrenal cortex is not under pituitary regulation.

Alterations in carbohydrate metabolism in hypophysectomized patients have been minimal. When cortisone maintenance is adequate, both oral and intravenous glucose tolerance tests are normal. Symptomatic hypoglycemia does not develop. Insulin requirement for diabetes mellitus is not changed after hypophysectomy.

Perhaps one of the most disturbing sequelae of hypophysectomy is the development of diabetes insipidus. Impairment of renal concentrating ability

TABLE 4. OBJECTIVE REMISSION AFTER TRANSCRANIAL HYPOPHYSECTOMY IN FOUR PUBLISHED SERIES

Authors	Total no. of cases	No. of remission	Percentage of remission
Luft et al (44)	54	27	50
Pearson & Ray (45)	333	144	43
Boesen et al (46)	92	42	46
Peck & Olson (47)	44	16	36

causes polyuria and polydipsia. Lipsett & Pearson (50) suggested that the development of diabetes insipidus following hypophysectomy appears to be related to the degree of damage to the hypothalamic pars nervosa system. Thus, if the pituitary stalk is severed at a high level, permanent and severe diabetes insipidus results. If the stalk is cut close to the diaphragma sellae, resulting in less trauma to the stalk, there may be minimal impairment of renal-concentrating ability, and hence no significant polyuria.

MECHANISM OF REGRESSION AFTER ENDOCRINE ABLATION

The clinical benefits of endocrine surgery in women with breast cancer have been well recognized, but the mechanism(s) by which excision of endocrine glands induces regression of cancer remains to be elucidated. Tumor regression following endocrine ablation seems to be the result of the removal of circulating hormones that are "sustaining" the growth of the neoplasm. Some of the effects on the tumor of removing certain hormones may conceivably be related to other factors yet to be determined.

The influence of ovarian hormones on the growth and differentiation of the mammary glands has been studied in many species, but particularly in the rat and the mouse. In all mammalian species, the principal early effect of estrogens is ductal growth. In later life, estrogen also stimulates acinar growth. The mechanism by which estrogenic hormones produce their effects on the mammary epithelium is not clearly understood. All experimental evidence, however, suggests that their action is most probably dual. Indirectly, they stimulate the secretion of prolactin by the anterior pituitary; directly, they have an effect on mammary epithelium and connective tissue. In mammary carcinogenesis, estrogens can induce mammary cancer—through their direct effect on mammary epithelium—but they also maintain tumor growth, an action mediated by the anterior pituitary (51).

Thus, the beneficial effect of castration has generally been considered primarily the result of estrogen deprivation. It has been reported that estrogen administration in premenopausal women may sometimes accelerate the growth of breast cancer. In patients responding to surgical castration, administration of small doses of estrogens can exacerbate the disease (52).

Estrogen excretion in the urine following oophorectomy has been studied by many investigators. Almost uniformly they have reported an immediate and precipitous drop in urinary estrogen excretion to a low level after castration; but the level does not fall further, and in most cases returns to the preoperative level or above (53–55). Although clear-cut human evidence is still lacking, experiments in rats seem to suggest that estrogen deprivation induces a rapid decline in prolactin release, and hence tumor regression (56).

The mechanism by which adrenalectomy induces tumor regression is also not clearly understood. It has been thought that adrenalectomy removes critical amounts of steroid hormones similar to those found in the ovaries. It has been demonstrated that adrenalectomy in post-menopausal or surgically castrated women causes marked reduction, or, in some instances, disappearance of urinary excretion of estrogenic substances (57). Available clinical data seem to support the postulate and experimental evidence that breast cancer dependent on ovarian hormones should respond favorably to adrenalectomy. It is now known that patients obtaining a beneficial response from castration are often benefited again by adrenalectomy, whereas oophorectomy failures frequently also fail to respond to adrenalectomy.

Whether the effects of adrenalectomy are indeed mediated by a decrease in circulating estrogens has yet to be proven conclusively. There has been no conclusive evidence that the human adrenal glands secrete estrogens. Recent studies, however, indicate that the major steroid hormone secreted by the human adrenals is dehydroepiandrosterone, which may be a precursor of estrogens (58). It is quite conceivable that adrenalectomy removes the major estrogen precursor in women without ovarian activity.

Suppression of adrenotropic, gonadotropic, and thyrotropic activities as a result of hypophysectomy induces profound effects on the adrenals, gonads, and thyroid. In patients with intact ovaries and adrenals, the beneficial effects of hypophysectomy may be the result of inactivity of these endocrine glands, comparable to their surgical ablation.

Hypophysectomy, however, also eliminates growth hormone and prolactin. In experimental studies, there is overwhelming evidence that prolactin is essential for maintenance of mammary tumor growth. The effect of estrogen, in these experiments, appears to be indirectly mediated through the pituitary, to increase prolactin secretion. No similar evidence is available in human studies for two major reasons: (*a*) No pure human prolactin has yet been isolated. Li et al (59) showed that highly purified human pituitary growth hormone also contains potent lactogenic activity. (*b*) No satisfactory assay is available for determining prolactin levels in human subjects.

Human growth hormone and ovine prolactin have been investigated for their ability to stimulate the growth of human breast cancer, but the results are negative (60). Using Li's highly purified human pituitary growth hormone, we have recently studied its effect in two women with advanced breast cancer. No activation of cancer has been observed in either of these two patients after prolonged administration for a period of five weeks. It has also been reported that hypophysectomy performed in patients who previously had had adrenalectomy did not produce any further regressions of cancer (61, 62). Thus, the effects of growth hormone and prolactin on human breast cancer are still not clearly defined.

HORMONE STUDIES IN HUMAN BREAST CANCER

Endocrine ablations for breast cancer, adrenalectomy and hypophysectomy in particular, are major operative procedures that produce regression of cancer in about half the patients with advancing disease. Thus, if response to endocrine ablation could be predicted, unnecessary major surgery would be avoided for those who could obtain no remission.

During the past two decades, renewed and greater efforts have been made at studying urinary excretion of steroids, including estrogens, pregnanediol, 17-oxosteroids, and 17-hydroxycorticosteroids. The studies were done with at least three purposes in mind: (*a*) to define the hormonal environment in which the disease evolves; (*b*) to elucidate the mechanism of tumor regression after endocrine ablations; and (*c*) to discover a method for predicting response to endocrine ablations.

Review of the literature dealing with hormone excretion in relation to human breast cancer provides few definite conclusions. Problems concerning the validity and interpretation of urinary steroid data are apparent: (*a*) excretion levels of hormones do not necessarily represent secretory activity; (*b*) variations in excretion rate are often measurements of the degree of illness of a patient rather than different endocrine environments related to the presence of cancer; and (*c*) the reliability of the methodology is often in question. Even so, the sophistication of recent methodology and a better understanding of cancer biology have led to the emergence of some interesting studies that are worthy of a brief discussion.

Urinary estrogens.—Dao (63) was the first to report that women with breast cancer still excrete significant amounts of estrogens, as determined by bioassay, after eradication of ovarian function by surgical excision, but that removal of the adrenals causes complete disappearance of estrogens from the urine of these patients. This observation suggests that adrenalectomy induces regression of cancer by removal of estrogens. It was subsequently reported that the level of estrogen excretion in urine could be correlated with clinical response to adrenalectomy. In that (64) study, it was shown that women with higher estrogen titers in urine respond better to adrenalectomy than do those who excrete only small amounts of estrogenic substances (Fig. 2).

Birke et al (65) studied estrogen excretion by the method of Brown in 19 postmenopausal women undergoing oophorectomy and adrenalectomy, and reported a lack of correlation between response to adrenalectomy and estrogen excretion level. Jull et al (66), on the other hand, gave data to support the earlier findings of Dao & Huggins that remission of breast cancer following adrenalectomy was associated with higher estrogen levels.

Urinary 11-deoxy-17-oxosteroids and 17-hydroxycorticosteroids.—Bulbrook and his co-workers (67) determined urinary 17-oxosteroids, including dehydroepiandrosterone, androsterone, etiocholanolone, and the 11-oxygenated 17-oxosteroids, and also 17-hydroxycorticosteroids (17-OHCS), before surgery, in an attempt to predict response to adrenalectomy or hypophysectomy. Of these urinary metabolites, determined in a series of 65 patients, only the 17-hydroxycorticosteroids and etiocholanolone were of use in predicting response to endocrine ablations. The mean 17-OHCS level was higher and the mean etiocholanolone level was lower in the failure group than in the remission group. From the ratios between 17-OHCS and etiocholanolone, a formula known as a discriminant function was devised: 80–80 [17-OHCS (mg/24 hr)+etiocholanolone (μg/24 hr)]. These authors reported that positive values of the function are correlated with good response to treatment and negative values with no benefit. They suggested that a negative discriminant was a contraindication to the use of adrenalectomy or hypophysectomy, and that there was a 50 percent chance of favorable response if endocrine ablation was done in patients with positive discriminants.

Studies confirming the work of Bulbrook's group are not available in the literature. Work in our laboratory (unpublished) failed to confirm Bulbrook's data. Miller et al (68) subsequently reported that a simple ratio between total 17-oxosteroids and 17-OHCS gave an alternative function that was in good agreement with Bulbrook's discriminant function.

Bulbrook and his colleagues have subsequently reported a statistically significant difference between controls and women admitted to hospitals for radical mastectomy for early carcinoma (69). They claimed that very few healthy women, but about 2/3 of the early breast cancer patients, gave negative discriminants. They suggested that a defect in androgen production was etiologically associated with clinical appearance of breast cancer, and that this defect could be detected before the appearance of breast cancer. This notion was promptly refuted by Wade et al (70) who concluded from their own study that the discriminant is not specifically affected in early breast cancer.

The discriminant function studies of Bulbrook et al had been criticized earlier by Nabarro (71) on the basis that the discriminant did not discriminate between two types of advanced carcinoma, but rather between more serious and less serious ills. Recent work by Zumoff et al (72) led to the conclusion that low levels of urinary 17-ketosteroids in patients with advanced

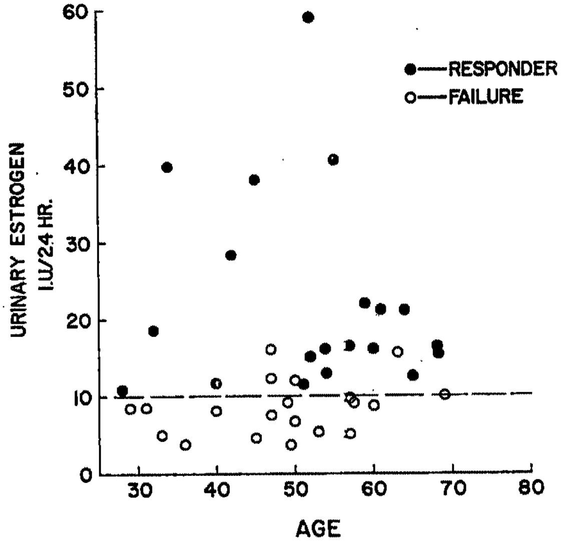

Fig. 2. Urinary estrogen and response to adrenalectomy.

breast cancer are a nonspecific consequence of illness. There was no evidence whatever to support the postulate that there is a decreased secretion of androgens in breast cancer patients.

Estrogen receptor protein in breast cancer tissue.—In recent years, studies of intracellular macromolecules with high and stereospecific affinity for estrogens have revealed the existence of specific protein "receptors" in estrogen-sensitive tissues, such as those of the uterus, vagina, and anterior pituitary (73) and, of particular interest, in mammary tumors from rats and human patients (74, 75). Studies carried out with experimental hormone-dependent mammary tumors, such as those induced in Sprague-Dawley rats by chemical carcinogens, showed that these tumors resemble the uterus and vagina in their affinity for estradiol, which was incorporated without chemical transformation. These studies also showed that the uptake of estradiol by these tumors varies; some were high and many were very low, an observation suggesting that the level of binding was related to the hormone sensitivity of the tumor. Jensen et al (76) reported that slices of hormone-dependent mammary tumors, on exposure to solutions of tritiated estradiol in vitro, showed a marked uptake of radioactive hormone, whereas tumors that did not regress following oophorectomy showed very little radioactive hormone uptake.

Soon after the original observations of the striking affinity of estrogens for target tissues, Folca et al (77) injected tritiated hexestrol into breast cancer patients undergoing adrenalectomy, and observed that there was a significantly greater uptake of radioactive hormone by the tumor than by the skeletal muscles in four patients who responded to the operation, but not in those who did not. These studies, together with the results obtained with experimental rat mammary tumors, suggest that measurement of receptor contents of breast cancer tissue in human patients may provide a valuable means for predicting response of the patients to adrenalectomy.

Jensen and his co-workers (78) undertook an investigation to determine whether estrogen binding by breast cancer tissue could be correlated with regression of the cancers after adrenalectomy or hypophysectomy. The method employed was either uptake of radioactive estradiol by the tumor tissue, or else density gradient ultracentrifugation, which sediments the estradiol-receptor complex as a well-defined 8S peak. Jensen's group studied 33 patients undergoing endocrine ablation. Tumors were negative for binding in 19 patients, positive in 11, and borderline in 3. Of the 19 patients negative for binding, 17 failed to respond to adrenalectomy; of the 11 positive for binding, 5 had remissions; and all 3 borderline cases were failures. These results suggest that tumors that lack the receptor proteins often fail to respond to endocrine ablations. Extended studies are necessary to determine the validity of these results.

Conjugation of steroid hormones by breast cancer.—Over the last few years, convincing evidence has been gathered that certain tumors contain various enzyme systems that catalyze metabolic transformations of steroid hormones. Of special interest are studies of the capacity of tumors to synthesize sulfate esters of sex hormones, and apparently to convert certain steroid precursors into physiologically active hormones.

The presence of enzyme systems for forming steroid sulfate esters in mammary gland and mammary cancer tissues was demonstrated by two groups of investigators (79, 80). In one study, Dao & Libby demonstrated that the breast cancer tissue contained two populations of cells, one that lacked enzyme, and one that possessed enzymes capable of forming sulfate esters with several steroid hormones. These authors also observed that the pattern of conjugation of steroid hormones is not the same in breast cancer tissue as it is in the normal liver tissues. The abilities of the breast cancer tissues to conjugate estrogenic and androgenic hormones vary from one individual to another. Some breast cancers have enzyme systems that synthesize estrogen sulfate more efficiently than they do dehydroepiandrosterone sulfate, whereas the case with others may be the reverse.

These findings led to a study to determine whether the formation of steroid sulfates in mammary cancer is related to the response to endocrine ablation in patients with advanced cancer of the breast. We reported earlier (81) that if breast tumors did not possess enzymes for the synthesis of steroid conjugates, they would not regress after adrenalectomy. In that study, tumors from 13 of the 44 patients were found to lack sulfotransferase for the synthesis of steroid sulfate, and all 13 failed to respond to adrenalectomy. Of the 10 patients whose breast cancers contained enzymes that synthesized sulfate dehydroepiandrosterone more efficiently than they did 17β-estradiol (i.e., the ratio DHEA:17β-E was greater than unity), 9 had objective remissions, and only 1 was a failure after adrenalectomy. Thus, of the 20 patients whose ratios of DHEA sulfate to 17β-E sulfate were less than unity, 19 failed, and only 1 responded to adrenalectomy. The remaining patient who had a ratio of unity for DHEA sulfate to 17β-E sulfate responded favorably to adrenalectomy. These results strongly suggest that sulfation of steroid hormones by breast cancer is related to hormone dependency of the tumor. The clinical correlation has thus far been remarkable.

In an extended study, we have further confirmed the earlier data (82). Table 5 shows data clearly demonstrating the correlation between the results of the in vitro study and clinical response to adrenalectomy. Perhaps the most striking observation is the finding that patients whose tumors do not possess sulfotransferases uniformly fail to respond to adrenalectomy, and all are dead (83). In contrast, the survival rate in patients having tumors that form more DHEA sulfate than estradiol sulfate is significantly greater.

If sulfation of steroid hormones by breast cancer is indeed related to the hormone dependency of a tumor, the biological mechanism of this relationship must be elucidated. It should be particularly interesting, from the clinical standpoint, to know the following: (*a*) whether conjugation of steroid hormones by breast cancer tissue has any prognostic value, namely its relationship to the occurrence of metastases; (*b*) whether determination of the

TABLE 5. SULFOKINASE ACTIVITY IN TUMOR AND RESPONSE TO ADRENALECTOMY

No. of patients	Sulfokinase activity in tumor DHEA:E-17β Ratio	Response to adrenalectomy	
		Remission	Failure
30	No activity	—	30
46	<1	6 (15%)	40
29	>1	21 (73%)	8
4	=1	3	1
Total 109		30	79

ability to conjugate steroid hormones in a primary breast cancer can predict future response to adrenalectomy or oophorectomy when metastases appear; and (*c*) whether metastatic lesions from different sites differ in their ability to conjugate steroid hormones.

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IMMUNITY TO NEUROBLASTOMAS AND MELANOMAS[1]

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INTRODUCTION

This article will review studies on immune reactions to tumor-associated antigens (TAA) of two types of human neoplasms, neuroblastomas and malignant melanomas. The reason for choosing these tumors is that they belong to the few human neoplasms which undergo occasional spontaneous regressions (indicating that they may be immunologically rejected by their hosts), and that they are among those most investigated with respect to both cell-mediated and humoral immune reactions against TAA. Knowledge provided by studying immunity to these neoplasms may help us to understand better host immunological defenses against other human tumors as well.

Rather than trying to make a chronological account of what information has been obtained so as to establish "priorities," we will discuss some of the principal findings which have evolved. Clinical evidence for host-mediated immune reactions against neuroblastomas and melanomas will be presented, and observations will be reviewed which show cell-mediated immune reactions against these tumors. We will summarize experiments demonstrating how lymphocyte-mediated destruction of neoplastic cells can be thwarted by blocking factors present in sera from tumor-bearing patients, and we will discuss reports on humoral antibodies to melanomas and neuroblastomas, detectable by immunofluorescence and cytotoxic tests. The nature of the antigens involved will be considered. Finally, we will speculate on what implications these immunological findings may have in relation to the three practical goals of cancer research: prevention, therapy, and diagnosis.

CLINICAL EVIDENCE FOR A HOST DEFENSE AGAINST NEUROBLASTOMAS AND MELANOMAS

Neuroblastomas and melanomas are among those few human neoplasms which have been reported to regress occasionally, completely or partially, in the absence of therapy considered adequate for elimination of all neoplastic

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cells. Everson & Cole who, in 1966, summarized 176 known cases of spontaneous tumor regression, list 29 authenticated regressions of neuroblastomas and 19 of melanomas. In all such regressions, it was considered unlikely that the therapy instituted (mostly surgery) could possibly have removed (or killed) all tumor cells (1).

A remarkable finding is that the spontaneous regressions recorded have often been dramatic leading to the rapid disappearance of large tumor masses. This implies that should the mechanism of regression be immunologic—which we tend to believe, as will be discussed later—the tumor-destructive power of a properly sensitized host is much larger than that detected in standard animal experiments designed to demonstrate tumor-specific transplantation antigens by rejection to neoplastic cells by immunized hosts (2).

Regression of neuroblastomas.—The frequency of regression of neuroblastomas varies between different clinical series from a conservative figure of 1–2 percent (3) up to 16 percent (4). It shows an interesting age distribution with regression being relatively common in children less than two years old at the time of diagnosis but extremely rare later (1). Once a neuroblastoma has regressed, it rarely comes back, and the patients remain healthy without neurological or other symptoms (1).

So-called neuroblastomas in situ are of great interest (5). Such tumors, localized in the adrenals, have been found at autopsies in approximately 1 percent of children less than three months old, who have died from causes other than neuroblastoma. Since the frequency of clinically overt neuroblastoma is approximately 1 case in 10,000 (6), a large number of the early occurring tumors must not become clinically detectable.

Regression of melanomas.—Complete and permanent regressions of melanomas are relatively rare, the incidence in several series being less than 1 percent (1). However, partial and temporary regressions are seen relatively frequently (5–15 percent). Regressions are not more common in any particular age group (1).

There are at least two remarkable features of the reported cases of spontaneously regressing melanomas. First, a depigmentation of the normal skin adjacent to the regressed melanomas has been seen in several patients (7). This may suggest that the regression may have been caused by some event destructive to normal pigment cells as well. Alternatively, the normal pigment cells may have been destroyed as "innocent bystanders," or they may not have been really normal after all. Second, there is some suggestive but very inconclusive clinical evidence that immunotherapy with plasma, blood, or lymphocytes from cured patients can be beneficial. A few striking examples may be worth mentioning: One patient who had melanoma with subcutaneous metastases became tumor-free, following inoculation of 250 ml blood from another patient whose melanoma had disappeared spontaneously

(8). Another patient had two temporary remissions of melanoma, metastasized to lung and brain, following two episodes of treatment with plasma from another patient who had been tumor-free 10 years after therapy for melanoma (9). A third patient with metastasized melanoma had undergone a spontaneous remission shortly following blood transfusion (10). Furthermore, inoculation of leukocytes from patients immunized against melanoma cells has occasionally led to tumor regression (11, 12), and intradermal immunization with melanoma cells has been reported to be therapeutically beneficial in some patients (12). In most cases, however, plasma, blood, or lymphocyte transfusions from patients cured of melanoma have not influenced the clinical course, and the outcome of the immunization trials has been variable. Thus, the effect of immunotherapy—as so far carried out—is difficult to assess.

Immunological destruction or differentiation?—Whether spontaneous tumor regression is due to destruction of the neoplastic cells or to their differentiation into more normal ones is unknown. There is suggestive evidence that some neuroblastomas can differentiate into benign ganglioneuromas (13). It is difficult to know, however, whether the malignant cells really became normalized, or whether the ganglioneuroma cells detected following regression were those that remained after the neuroblastoma cells had been killed off. In view of some work done with mouse mammary carcinomas, the latter alternative may appear more likely: it was originally believed that mammary carcinoma cells may differentiate into their normal counterparts in a host immune to their TAA. Later studies showed, however, that the normal mammary gland cells were just selected for by an immunological killing of the neoplastic ones (14).

Lymphocyte infiltration.—It has been reported that more lymphocytes are seen within the more differentiated neuroblastomas—which are those with a better prognosis—than in the more anaplastic ones (15). This finding may be reminiscent of the demonstration that histologically detectable lymphoid reactions to carcinomas of the breast often imply a favorable prognosis (16). It does not prove, however, that the lymphocytes detected in the more differentiated tumors play any causative role in destroying the neoplastic cells in vivo. The observation that patients with high peripheral lymphocyte counts at the time of diagnosis generally do better clinically than those with low counts (17) also supports the notion that immune factors are involved in patient resistance to neuroblastomas.

Lymphocyte infiltrations into melanomas are common (18–20), and lymphoblasts are often seen in the regional nodes. Lymphocyte and plasma cell infiltration into the tumors often indicates a favorable prognosis (18). This suggests that an immunological response may be operating in those patients whose melanomas regress.

In conclusion, it seems fair to state that there is some clinical evidence

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for a host immune defense against neuroblastomas and melanomas which is occasionally capable of causing the "spontaneous" regression of these tumors. However, the regressions might, instead of being immunological, be due to some intrinsic characteristics of the neoplastic cells. Evidence to be presented below appears to favor the former explanation.

EVIDENCE FOR CELL-MEDIATED IMMUNITY TO NEUROBLASTOMAS

Neuroblastomas were among those human tumors in which a cell-mediated immunity to TAA was first detected (21, 22). A colony inhibition test (23) was used. It had been previously employed in studies on animal tumor immunity and there shown to be capable of demonstrating lymphocyte-mediated inhibition of neoplastic cells (24–26). The principle of the test is to cultivate tumors in vitro, seed a small number of their cells into petri dishes, and expose them, the following day, to immune (and control) lymphocytes. This way, one can demonstrate that specifically immune (versus control) lymphocytes decrease the formation of tumor colonies in vitro. The colony inhibition test has been recently modified to a cytotoxicity assay (23), in which Microtest plates rather than petri dishes are used and individual cells instead of colonies counted; the amount of target cells and lymphocytes needed can thus be reduced and the counting procedure simplified.

By using the colony inhibition technique on neuroblastoma cells obtained at surgery and cultivated in vitro, it was possible to demonstrate that blood lymphocytes from patients with neuroblastoma were inhibitory to plated autochthonous and allogeneic neuroblastoma cells, while normal skin fibroblasts from the same patients were not affected (21, 22). A total of six autochthonous tumor cell-lymphocyte combinations were studied, all showing a specific tumor immunity. Allogeneic lymphocytes from neuroblastoma patients were reactive in 14 of 16 patients tested (22). This indicates that neuroblastomas possess a common TAA. The antigen is apparently absent from other types of human neoplasms, since lymphocytes from donors with tumors other than neuroblastoma were not reactive against neuroblastoma cells, and since lymphocytes from patients with neuroblastoma were not cytotoxic to other types of human tumors.

The degree of lymphocyte-mediated colony inhibition was found to be approximately the same when patients were studied who either had widespread disease or whose tumors had been successfully removed (22). This may appear paradoxical but is similar to observations made with animal tumors (24–28). In order to detect minor differences in lymphocyte reactivity between the two groups, titrations of the effect of various doses of lymphocytes are needed; such titrations have not been carried out in the neuroblastoma tumor system (and not in the melanoma system either).

Lymphocytes from relatives of neuroblastoma patients (mothers, brothers, sisters, fathers) were often found to be inhibitory to the colony formation of neuroblastoma cells but did not affect normal skin fibroblasts from the tumor donors (21, 22). Since the mothers' lymphocytes were found to be reactive on neuroblastoma cells from children other than their own, and since no such reactivity was seen with control lymphocytes from the mothers of children not having had neuroblastoma, it seems likely that the "reactive" mothers were, indeed, immune to some antigen(s) associated with neuroblastoma cells and not only to the normal alloantigens of their own children. These findings are similar to independently made observations with human sarcomas, studied by immunofluorescence and complement-fixing techniques (29).

A cell-mediated immunity to common antigens of neuroblastoma cells has also been detected in an assay which measures tritiated thymidine uptake in the target cells rather than colony formation (30). Since the end results come out from a scintillation counter, thus eliminating time-consuming counting under the microscope, this technique may be more adaptable to large-scale testing programs.

EVIDENCE FOR CELL-MEDIATED IMMUNITY AGAINST MELANOMAS

Findings on cell-mediated immunity against melanomas have been quite similar to those obtained with neuroblastomas. Lymphocytes from patients with melanomas are colony inhibitory (and cytotoxic) to cultivated melanoma cells, independently of whether these are of autochthonous or allogeneic origin (31, 32). In one published study (32), lymphocytes from seven patients with melanoma were tested against autochthonous tumor cells, and lymphocytes from 25 patients with melanoma were tested against allogeneic melanoma cells. A cytotoxic effect on melanoma cells was seen with lymphocytes from five of the patients belonging to the first group and all of those in the latter. Normal skin fibroblasts from the donors of the target tumor cells were not affected and neither were cells from other tumors, such as carcinomas of the breast, colon, lung, or sarcomas. Lymphocytes from patients having tumors other than melanomas did not affect melanoma cells.

As in the experiments with neuroblastomas, lymphocytes from patients with growing melanomas have been found to be reactive against melanoma cells established in culture prior to the tests, the degree of reactivity being approximately the same as that seen with lymphocytes from patients who are clinically tumor free (32).

The original observations on cell-mediated immunity to melanomas (31, 32) have been confirmed in other laboratories and with other techniques (30, 33–35). Thus, reactions against melanoma cells from allogeneic (as well as autochthonous) donors have been detected by isotope assays measuring tritiated thymidine uptake in the target cells (30, 36), or Cr^{51} release from them (34), and evidence for a strong immunological reaction against melanomas was obtained in studies measuring tritiated thymidine uptake in lymphocytes from melanoma patients upon exposure to melanoma extracts, while transformation was not seen with control lymphocytes (35).

It was observed in a recent study that some patients were repeatedly more reactive when tested with one set of melanoma target cells than with another, while the situation was the reverse for other patients (34). This indicates—as will be further discussed below—that melanomas have more than one TAA and that these antigens often, but not always, overlap.

A different type of evidence for cell-mediated immunity against melanomas has come from investigations showing that delayed hypersensitivity reactions can be elicited in melanoma patients, using extracts of melanoma cells (37–39). In the original study (37), such reactions were seen in all of three patients with localized disease, but in none of five patients with widespread disease; later work (38), however, has not fully held up the differences between these two patient groups. Reactions to autochthonous (37, 38) as well as allogeneic (39) melanoma extracts have been detected, thus confirming the cross-reacting nature of at least some of the antigens involved. An interesting observation made during this study was that reactions could be elicited with normal skin extracts in some of those patients who reacted with the melanoma extracts (38, 39). This may suggest that normal skin extracts contain antigen(s) cross-reacting with those in the melanomas; however, the findings should be interpreted with caution because of the possibility that some of the effects observed in this kind of work are nonspecific.

Before concluding this section, we want to point out that existing knowledge on cell-mediated antitumor immunity is still scarce with respect to the individual roles of the different cell types involved (lymphocytes of various types, macrophages). The in vitro assays employed have primarily concerned the ability of sensitized lymphocytes to kill target tumor cells and are, therefore, not likely to reflect the entire situation in vivo: if, for example, there would be a scarcity of certain inflammatory cells necessary for tumor rejection in a cancer patient, his lymphocyte response according to the in vitro tests may still be intact while the rejection of antigenic tumor cells in vivo may be hampered. Evidence that such defects can occur has come from studies on tumor-bearing patients' ability to mount delayed hypersensitivity reactions against certain antigens (40, 41), as well as from investigations on tumor resistance in guinea pigs bearing transplanted hepatomas (42). This area deserves more study, and methods, such as the macrophage migration inhibition assay, the skin window technique, etc., may complement those already used, as they have in studies performed with other kinds of tumor (43–45).

DEMONSTRATION OF SERUM-MEDIATED INHIBITION OF THE CYTOTOXIC EFFECT OF LYMPHOCYTES IMMUNE TO TUMOR-ASSOCIATED ANTIGENS

Blocking serum effects.—The finding that lymphocytes from patients with growing neuroblastomas and melanomas are cytotoxic to cultivated tumor cells of the respective types is analogous to observations made in experimental animals (24–28, 46–49). In the animal systems, it has been shown that serum from the tumor-bearers can specifically block the lymphocyte effect, while serum from animals whose tumors have regressed or have been removed does not block (24, 25, 27, 28, 46–49). It has, therefore, been postulated that blocking serum factors play a major role in facilitating tumor growth in vivo (25). This postulate is supported by work showing that sera from tumor-bearing animals which can block lymphocyte reactivity in vitro will facilitate ("enhance") tumor growth when inoculated in vivo (28, 50, 51); that eluates from tumors growing in vivo can block lymphocyte reactivity in vitro (28, 51) and enhance the growth of tumor transplants in vivo (50, 52); that animals with blocking sera (and immune lymphocytes) are at least as susceptible to tumor grafts of the respective types as are untreated controls (or even more so) (28); that animals whose blocking factors have disappeared following tumor removal, but which have the same degree of cell-mediated tumor immunity as those whose neoplasms have been removed (28, 49), are resistant to grafts of the respective tumors (28); and that animals which have been splenectomized have a decreased blocking serum activity (49) and a heightened resistance to grafted tumors of the respective types (53).

Of course, a primary tumor is, under natural conditions, not likely to start growing just because blocking factors are there. These factors probably are antigen-antibody complexes (54), the antigenic part of which is contributed by the tumor, and the antibody part being formed by the host upon stimulation by antigen released from the tumor. As soon as the blocking factors are present, however, they are likely to facilitate tumor growth for the reasons given.

On the basis of knowledge obtained from the animal models, experiments were performed to search for serum-mediated blocking of lymphocyte cytotoxicity against neuroblastomas and melanomas. Sera from each of four patients with growing neuroblastomas could block lymphocyte-mediated cytotoxicity against neuroblastoma cells, while sera from none of six tumor-free patients could (22). No blocking was seen with sera from the mothers of the neuroblastoma patients; the lymphocytes of these mothers were cytotoxic to neuroblastoma cells. Sera from 13 of 15 patients with growing melanomas blocked the cytotoxic effect of lymphocytes from melanoma patients (55). Blocking was also obtained with sera from two of seven patients who were tumor-free (55), but both of these patients later had relapses (56). The blocking effect was specific: Sera from patients with other tumors did not block and sera from melanoma patients had no blocking activity when tested with other target cells.

The blocking activity could be seen independently of whether sera were tested from the same patients as the lymphocytes or from other patients with the same diagnosis (55). However, it appeared to be greater when both lymphocytes and sera came from the same patients (55). These data would support the postulate that melanomas have more than one set of TAA, one common and one individually specific, autochthonous lymphocytes being able to react against both and their reactivity being best abrogated by autochthonous sera containing blocking factors against both specificities.

The demonstration of a blocking serum effect is interesting for at least two reasons. First, it strengthens the conclusion that the cell-mediated immunity detected is directed against a TAA, even when allogeneic patient-target cell combinations are studied (24). One would have expected that, if the in vitro reactions were just directed against normal alloantigens or were of a nonspecific nature, sera from some patients with tumors of other types also should have blocked.

Second, the blocking activity correlates well with the clinical tumor status of the patients (55, 56). This indicates that the blocking effect in man, as in animals, plays an important role in facilitating tumor growth in vivo (25, 27, 28). The latter conclusion is supported by the finding that a blocking serum activity could be repeatedly detected before tumor recurrence (55, 56). Additional evidence to the same end comes from studies on certain patients who were closely followed over a long period. One patient, for example, whose metastasized melanoma regressed spontaneously, had a blocking serum effect as long as the tumor grew. The effect disappeared before the remission was detectable, after which it has stayed away (56).

One may still argue that the blocking activity is the result rather than the cause of progressive tumor growth, and that it does not affect tumor behavior in vivo. Although this argument is difficult to reject completely in the human situation, it has been made very unlikely as an explanation of the animal findings (25, 28), for reasons already given.

Unblocking serum activity.—Mice, whose Moloney virus-induced sarcomas have spontaneously regressed, were found to have so-called "unblocking" antibodies, which can counteract the blocking effect of sera from mice with progressively growing such tumors (27, 57). Similar unblocking effects have been observed with sera from rats whose polyoma tumors have been surgically removed (58). Since there is evidence (27, 28, 57–60), to be discussed below, that inoculation of unblocking sera can be therapeutically beneficial in vivo, searches for unblocking antibodies to human tumors are particularly interesting.

Tests made for unblocking activity of sera from patients whose tumors had been surgically removed, or were in a state of remission, have shown that sera from certain patients who had become tumor-free could cancel the blocking effect of sera from patients with actively growing neoplasms of the respective kinds, the unblocking effect being specific (61). These findings suggest that a monitoring of unblocking serum activity in patients treated in various ways may yield important information as to the defense against their tumors. One may speculate that the unblocking titers decrease preceding tumor recurrence considerably earlier than blocking serum effects can be detected. The possible implications of the findings for immunotherapy will be discussed below.

Evidence for Cytotoxic and "Fluorescent" Antibodies Against Melanomas and Neuroblastomas

Cytotoxic antibodies.—Lewis et al have shown that sera from patients whose localized melanomas have been surgically removed are cytotoxic to cultivated melanoma cells from the same patients in the presence of complement (62). Virtually no cross-reactivity with melanoma cells from other patients was found. Cytotoxic antibodies were not detected in patients with widespread disease. They were shown to decrease in titer as melanomas grew larger, and to return upon tumor removal.

Too few studies on cytotoxic antibodies have been performed with the colony inhibition and microcytotoxicity assays to give a clear picture as to the degree of cross-reactivity among melanomas detected by these methods. Available data suggest, however, that cross-reactions do, indeed, occur (31, 33), as also indicated from studies on blocking (55) and unblocking (61) serum activities (see the preceding section). Likewise, cross-reactions have been observed between neuroblastomas, when sera were studied with the colony inhibition test for cytotoxic or blocking activities (21).

Antibodies detected by immunofluorescence tests.—Following Klein's pioneering studies on Burkitt lymphomas with the membrane immunofluorescence test (63), several investigators have been able to demonstrate TAA in human melanoma cells using immunofluorescence techniques (33, 34, 62, 64–66). In contrast to Klein's work on Burkitt lymphomas, most assays carried out on melanomas have been done with fixed material and have detected a cytoplasmic antigen. Patients with malignant melanomas and, to a much less extent, healthy controls and patients with other types of tumors, have antibodies reacting with an antigen which is common to many melanomas. In addition, there is some evidence also for a surface-localized antigen in melanomas, detectable by studying living cells (33, 34, 62, 65). This antigen is individually unique for each melanoma according to Lewis et al (62), while findings from some other groups (33, 34, 65) indicate that melanomas have a cross-reacting, surface-localized TAA; most likely both the common and unique antigens are real (34, 55), and are detected under slightly different experimental conditions.

The Nature of the Tumor-Associated Antigens of Neuroblastomas and Melanomas

The TAA are not alloantigens.—Our knowledge of the nature of the TAA of any human neoplasm is quite insufficient. However, the cell-mediated and humoral immunity detected against autochthonous tumor cells cannot, by definition, be ascribed to alloantigenic differences. That the effect detected when allogeneic lymphocytes and sera are used is due to such differences can also be excluded. If it were so, one would not have expected the immune reactions to be regularly observed with lymphocytes from patients having the respective types of tumors, but not with lymphocytes from patients with other kinds of tumors or from healthy subjects, when the same lymphocyte suspensions are used in a criss-cross pattern as experimental material with one set of target cells and controls with another (32, 55). Furthermore, normal cells from the donors of the target tumor cells have not been affected.

Are the TAA normal tissue antigens?—It is more difficult to settle whether the TAA are tissue-(or organ-) specific rather than tumor-specific, particularly since skin fibroblasts (and not nerve cells or normal pigment cells) have been used as control material for neuroblastomas and melanomas.

One may argue that the former possibility is decreased by the good correlation established between the in vitro data and the patients' tumor status in vivo, if the blocking effect of serum from tumor-bearing patients is taken into account (55), and that such a correlation would not exist if the immunity detected could not, in the absence of blocking factors, destroy neoplastic cells in vivo. The demonstration of cell-mediated immunity to neuroblastomas in certain relatives of neuroblastoma patients further argues against the supposition that the antigens are just tissue-specific, since these relatives, who had no blocking factors, showed no signs of autoimmunity (22). Neither was any autoimmunity seen in patients cured of neuroblastomas whose lymphocytes were reactive on the tumor cells.

On the other hand, the demonstration that patients with melanomas had delayed hypersensitivity reactions against normal skin extracts (in 6 of 9 patients tested in one study), as well as to the melanoma extracts (36, 37), might be interpreted to indicate that there is an antigenic cross-reactivity between neoplastic and normal cells of the same tissue. Even if this is true, one still must remember that the reactions against these antigens gave delayed hypersensitivity in vivo (generally believed to be correlated with a tumor-destructive ability) and led to tumor cell destruction in vitro, implying that the antigens involved did not at all appear as "self" to the organism and thus may be useful for tumor prevention and therapy.

In order to clarify the in vivo role of immunity to the common TAA of neuroblastomas and melanomas—as well as of other human neoplasma (32) —it is important to find out whether common TAA of the corresponding "tissue-specific" type can act as tumor-specific transplantation antigens in vivo. This may be studied by searching for tissue type specific TAA in murine melanomas, and also in such animal neoplasms as carcinomas of the bladder, colon, and breast, and then to study whether animals in which a cell-mediated immunity to them (but no blocking) has been induced, are specifically resistant to tumor grafting, as well as to the induction of new neoplasms of the respective types (see further discussion in a subsequent section). Obviously, these animals should be studied for evidence of autoimmunity to normal cells as well.

A possible carcinoembryonic or viral nature of TAA.—There is suggestive evidence that common TAA detected in human colonic carcinomas are of an embryonic nature (67, 68), and thus might be the result of either a genetic derepression or of the repressed formation of some molecules that would normally have masked the new antigens at the cell surface. It is as yet unknown whether the common TAA of neuroblastomas and melanomas may also be of an embryonic type.

An alternative explanation, which does not necessarily exclude the former one, is that the common TAA are caused by viruses, which may have either induced the tumors or become secondarily associated with them. The finding that lymphocytes from relatives of patients with neuroblastomas are reactive to neuroblastoma cells would support such an explanation (22). One does not know, however, if the reactivity observed with these lymphocyte samples is due to sensitization against a TAA, which is determined by a gene more common in the "neuroblastoma families" and expressed in some cells of the healthy relatives; in other words, these findings do not necessarily imply that an infectious agent is involved. It may be relevant in this context that the expression of detectable Gross (G) surface antigen of mouse (leukemic) cells has been shown to be inherited in a Mendelian way (69), the antigen also being detected in normal cells.

Individually unique TAA.—As already pointed out, there are some data indicating that malignant melanomas have individually unique TAA in addition to their common ones and that the same may be true for other human neoplasms as well. The respective roles of the individually unique as compared to the common TAA in host reactivity against cancer are unknown. There is increasing evidence that both common and unique TAA are present on chemically (70, 71) and known-to-be virally (72–75) induced tumors as well. However, animal experiments indicate that individually specific TAA play the dominant role as inducers and targets of immunological defense against chemically induced tumors (76), while the common ones are more important against tumors of a known viral etiology (77). It may be possible that the local deposition of large doses of potent chemical carcinogens favors the appearance of tumor cells which have strong individually specific TAA, and because of that, low expression of the common ones, in a way reminiscent of the demonstrated decreased amount of H-2 antigens in cells with strong TL antigens and vice versa (78).

The nature of the individually unique TAA is unknown. It may represent dissimilarities between different tumors due, for example, to random genetic changes during the induction and further multiplication of the neoplastic cells (2).

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Implications of the Immunological Findings for Tumor Prevention, Therapy, and Diagnosis

Prevention.—The demonstration of TAA common to each of the neuroblastoma and the melanoma groups might suggest that "vaccination" against these tumors may become feasible. However, conclusions as to whether this is a realistic goal would be premature at present. On the one hand, immunization against the common TAA might prevent tumors in the same way as SV40 virus tumor induction in hamsters can be thwarted by repeatedly inoculating the animals during the latency period with either infectious SV40 virus or SV40 virus-induced tumor cells (79, 80). On the other hand, the common "tissue type-specific" TAA observed in neuroblastomas and melanomas might not be able to immunize against tumor induction at all, or they may concomitantly immunize well enough against normal tissue antigens to induce autoimmune diseases. The latter possibility might prevail if the common TAA are closely related to antigens present in small doses in normal cells, for example, when they divide.

As already pointed out, even if small amounts of the TAA of neuroblastomas and melanomas would be ultimately detected on normal cells of the respective tissues (and we do not know if it will be so), this should not automatically reject the possibility that the same TAA could be useful for both tumor preventive and therapeutic purposes. The organism's response to both normal tissue-specific and tumor antigens involves a complex interaction between several different cellular as well as humoral components. On the one hand, it may be possible to tip the balance in favor of tumor destruction, even if the common antigens of the neoplastic cells are not qualitatively distinct from those of some normal cells. On the other hand, we must realize that an effective immunological prevention of tumors may be difficult to achieve even if their TAA would be entirely tumor-specific.

Some recent studies by Shier (81) may be relevant in this connection. He found that mice could be immunized against a synthetic antigen containing the presumed receptor site for wheat germ agglutinin (a lectin capable of specifically agglutinating tumor cells and normal dividing cells—but not normal cells that are not in mitosis). Mice immunized with the antigen in complete Freund's adjuvant had a transplantation resistance to tumor cells (carrying the receptor site), while there was no marked deleterious effect on biological processes in normal dividing cells. One may speculate (or at least hope) that even if the TAA of neuroblastomas and melanomas would also occur in minor amounts on normal cells, an immune response may be still capable of selectively affecting the tumor cells since these would have quantitatively greater expression of the antigens.

Therapy.—Before discussing this topic, we may consider, first, whether patients with melanomas and neuroblastomas really are suitable candidates for immunotherapy and, second, whether immunotherapy of human neoplasms should be done at all. The fact that melanomas and neuroblastomas disappear spontaneously can make a possible therapeutic success inconclusive. One may as well argue, however, that just because these tumors can regress, they are particularly suited for immunotherapeutic trials, since it may be easier to "tip the balance" favorably with these tumors than with neoplasms that never regress. If some initial success is obtained, very rigorous controls will, of course, be needed before one can exclude that only spontaneous regression has been observed.

One may argue that immunotherapy of human neoplasms should not be tried at the present time, since too little success has been obtained with primary animal tumors to justify the potential risks of a human trial. If, nevertheless, the decision will be to go ahead on a limited number of patients, it seems essential that the following criteria are met. First, the patients selected must be those with a terminal prognosis. Second, among these patients, the only ones in whom an immunotherapeutic trial would be meaningful are those with a relatively small tumor burden. Third, the patients should be carefully followed with such in vitro assays that can yield information relevant to the qualitative as well as quantitative immunological response to TAA (cell-mediated antitumor immunity, blocking, unblocking, and cytotoxic serum activities, general ability to mount delayed hypersensitivity reactions). If the patients are closely followed this way, and the results are published, information may be drawn even from experiments in which no curative effects can be discerned.

We will now discuss some ideas for human immunotherapy, suggested by animal experiments that have been carried out during the last few years.

"*Vaccination.*"—Perhaps the most logical way to go about the problem would be to try finding some "vaccine" which could greatly increase cell-mediated antitumor immunity, as well as the level of unblocking and cytotoxic antibodies, without creating any risks for the development of tumor-enhancing antibodies. Unfortunately, no such "vaccines" are available, neither against animal nor human tumors. Tentative ways for developing them (if at all possible) may involve the use of chemically modified tumor antigens, tumor cells attenuated by growth in tissue culture, etc. It does not seem likely, however, that anything practically applicable will appear along these lines until more is known about the nature of the tumor antigens and about the ways to achieve different types of tumor immunity.

An alternative way to "vaccinate" patients against their growing neoplasms would be to inoculate attenuated tumor cells intradermally, a procedure which has met with some success in experiments carried out in mice in which tumor transplantation immunity was induced (82), as well as in guinea pigs in which regressions of small established tumors could be achieved (83). Intradermal inoculation of melanoma cells has also been reported to lead to some regressions in human patients (12).

BCG.—Another approach, which has been tried with leukemias in particular, is to stimulate nonspecifically the immune response by inoculating Bacillus Calmette Guèrin (BCG) (84, 85), endotoxin, or other similar products. There is evidence that treatment with BCG might be therapeutically beneficial, especially in leukemia patients who have been given vigorous chemotherapy to reduce remaining tumor mass (86). In a few melanoma patients, complete tumor remission has been seen following BCG inoculation into subcutaneous metastases (87, 88). There is also evidence, however, that BCG treatment of animals with solid tumors can enhance tumor growth (89, 90). Extreme caution is, therefore, recommended before applying this mode of therapy to solid tumors. If BCG is tried, it should be given only to patients without blocking serum activity and one should monitor for appearance of blocking factors during therapy. One may also consider, first as a project for animal experimentation, the combined therapy with BCG and unblocking antibody inoculation, the latter aspect being discussed below. By such a combination therapy, it might be possible to achieve the stimulatory effect of BCG on cell-mediated immunity and, at the same time, to counteract its possible stimulatory effect on blocking antibody formation (as well as the blocking serum activity already present in the treated individuals).

Induction of delayed hypersensitivity to non-tumor antigen at the tumor site.—During the last few years, a way of treating skin carcinomas has been developed which has met with remarkable success and probably involves an immunologic component. Tumors are exposed to the focal application of chemical agents which are likely to elicit delayed hypersensitivity reactions, and this has led to disappearance of both the exposed tumors and, occasionally, of other tumors of the same type (91). Most likely, the cures obtained were the outcome either of a specific lymphocyte-carcinoma cell interaction or of a nonspecific destruction of carcinoma cells accompanying a specific reaction with the sensitizing antigen. It seems worthwhile investigating whether a similar therapy can be of any value in patients with cutaneous melanoma metastases.

Inoculation of lymphocytes.—The quite modest success so far obtained by inoculating allogenic, presumably immune lymphocytes, derived from cross-immunization experiments, may argue against a continuation of that particular line of work. However, it is possible that the transplantation of immune cells to cancer patients will have something to offer in the future. To this end, techniques have been developed in which large amounts of autochthonous (and allogeneic) patient lymphocytes can be obtained by cultivation of lymphoid cells in vitro (92); it must, however, be made certain that the lymphoid cells so grown are, indeed, capable of reacting against the TAA of the tumors involved. The chances that lymphocyte transfusion will become therapeutically useful may be increased if animal models are first devised in which graft versus host reactions can be controlled, in which the inoculated immune cells are not rapidly eliminated, and in which the blocking serum effect in the treated patients can be cancelled or overcome. It is worthwhile noting that, under optimal conditions, some immunotherapeutic success has been demonstrated in tumor-bearing mice and rats given lymphocytes immune to the specific antigens of the respective neoplasms (59, 93, 94).

Transfer factor.—Inoculation of transfer factor (95) may be worth trying, since transfer factor is believed to increase cell-mediated immunity specifically without increasing the formation of tumor-enhancing antibodies.

There are no good animal models available so the experiments must be carried out directly in man. It may be best to inoculate transfer factor from patients whose own tumors have either regressed "spontaneously" or been eliminated by conventional therapy. Healthy relatives of neuroblastoma patients with specifically cytotoxic lymphocytes may offer another source for preparation of transfer factor. It is not advisable to immunize actively patients (or healthy persons) for preparation of such factor because of the potential hazards involved in inoculating tumor cells (or extracts) into human subjects.

Neuraminidase.—Another procedure for experimental therapy is suggested by the recent demonstration that neuraminidase-treated tumor cells inoculated into tumor-bearing animals can sometimes induce regression of established tumors (96). The mechanisms of this phenomenon are unknown, as is the degree of success with which it may be applicable to primary, spontaneous neoplasms.

Splenectomy.—Splenectomy as a means of reducing blocking antibody formation may be worth considering (49, 53). Relatively little, however, is known about the value of splenectomy in individuals already bearing tumors, so more information from animal studies is needed before human experiments should be carried out.

Drug-induced depression of blocking antibody formation.—One can also speculate that some chemotherapeutic agents may decrease blocking antibody formation without comparably affecting cell-mediated tumor immunity There is preliminary evidence that treatment of mice with a small dose of cytosine arabinoside will decrease their blocking serum activity and, concomitantly, depress tumor growth, while treatment with a larger dose also depresses cell-mediated immunity and has no antitumor effect (97). Since the formation of unblocking and cytotoxic antibodies is likely to decrease as well following chemotherapy, the use of drugs for depressing blocking serum activity may be a two-edged sword. This is an area needing more research in experimental animals, as well as in patients undergoing chemotherapy. The influence of radiotherapy on the various immune parameters is also worthy of thorough studies.

Another area, which may have a great potential, is that of combining chemotherapy with immunotherapy in such a way that most neoplastic cells are killed by the drugs and the few remaining ones destroyed by immunological means (94).

Inoculation of sera with unblocking and/or cytotoxic properties.—Finally, it may be worthwhile to consider inoculation of unblocking and/or cytotoxic sera (27, 28, 46, 58, 59) taken from patients who have had spontaneous remissions or who have been cured by conventional therapy. By cytotoxic sera we mean sera that are cytotoxic in the presence of complement from the same species (human). The reason for this mode of therapy is that inoculation of unblocking sera can cure some mice of primary Moloney sarcomas (27, 59) as well as some rats of transplanted polyoma tumors (28, 58), and that it will also arrest the growth of primary polyoma virus-induced kidney sarcomas in rats (60). At least in the polyoma system, the tumor regressions observed are more likely due to unblocking than to cytotoxic serum effects (58), and they cannot be ascribed to the action of antivirion antibodies. It should be borne in mind, however, that "unblocking" sera might be capable also of "arming" lymphocytes so these become more capable of killing tumor cells, of opsonizing tumor cells, of recruiting more macrophages into the antitumor reaction by providing cytophilic antibodies, and of killing tumor cells in the presence of complement.

There are no data to indicate what in vivo effects unblocking sera may have against spontaneous neoplasms or against tumors whose antigens are of the tissue type-specific category, a problem which deserves thorough study. It may be worth recalling, however, that a few cases have been reported in which plasma from patients who had a particularly favorable course of neoplastic disease was inoculated into other patients with widespread tumors, and that improvement (including some complete regressions) was seen (1, 8–10). One of the more exciting reports in this connection is the recent demonstration that one patient with hypernephroma metastatic to the lung has so far remained tumor-free one and one-half years after the onset of biweekly transfusions of 500 ml plasma from another patient, cured of hypernephroma (98). Problems arise when trying to interpret all these data, however, since, in many cases, plasma inoculation has had no beneficial effects (1, 9), and since melanomas (as well as hypernephromas) occasionally regress in the absence of any treatment (1).

If inoculation of convalescent plasma or serum (from patients with spontaneous remission of melanoma, for example) should be tried in human patients, the following criteria have to be met: (*a*) the plasma infused should be known to be unblocking and should be given in sufficient amounts to abrogate the treated patients' blocking serum activity, as tested in vitro; (*b*) it should be given frequently enough to maintain the unblocked state; and (*c*) cell-mediated antitumor immunity as well as unblocking serum activity should be followed by appropriate in vitro methods. Treatment of a few patients under such well-monitored conditions may tell whether therapy with unblocking sera has any future. If the stipulated requirements for doing this kind of therapy are not met, however, and convalescent sera of unknown blocking activity injected, or the patients not followed with regard to changes in their blocking titer, it is likely that just more confusion will be added to the already rather confused story of cancer serotherapy. Even more important, there is a risk that the treatment would enhance rather than counteract tumor growth and so be clinically harmful.

To summarize this section, it seems likely to us that immunotherapy of melanomas and neuroblastomas has a future as an adjunct to other forms of therapy (surgery, radiation, drugs), particularly in patients who have small remaining tumor masses that cannot be destroyed otherwise. One must hope, however, that the development of sound modes of immunotherapy will not be hampered by unskilled approaches based on wishful thinking rather than on knowledge provided by preceding animal experimentation.

Monitoring of immune response in patients undergoing conventional therapy.—Even before immunology contributes to tumor prevention and therapy, it may indicate how already existing modes of therapy (surgery, radiation, drugs, hormones) can influence cell-mediated tumor immunity, as well as blocking, unblocking, and cytotoxic serum effects.

We already know that the blocking serum activity of tumor-bearing animals and of human cancer patients commonly disappears following successful surgery (28, 49, 55, 56), and that a blocking activity can be seen prior to clinically detectable tumor recurrence. More systematic studies of this phenomenon in man are needed, however. One also needs more knowledge of possible changes in unblocking serum activity in relation to tumor surgery (and tumor recurrence after surgery). Information derived from such studies might provide ways to monitor changes in a patient's immunological defense against his tumor before it is too late to institute appropriate therapy.

The great probability that some chemotherapy can decrease cell-mediated tumor immunity, as well as the recent demonstration that it can depress blocking serum activity (97), both prompt the use of in vitro methodology to follow the immunological effect of chemotherapy systematically. It is possible that modifications of existing schemes of chemotherapy, or a combination of chemotherapy with, for example, inoculation of frozen, immunologically intact autochthonous lymphocytes, or unblocking antibodies, may offer new therapeutic opportunities.

There are indications that some modes of radiotherapy can increase detectable immunological reactions against tumors: higher titers of antibodies to the membrane antigen(s) of Burkitt lymphoma cells have been seen in patients whose local Burkitt lymphomas were irradiated (99). One may expect that—under other conditions—radiotherapy will lead to a decreased immunological reactivity. Very little is known about the effects of different types of radiotherapy on cell-mediated tumor immunity, blocking serum activity, cytotoxic and unblocking antibody titers.

The techniques available today may be helpful in providing guidelines in this area. For studies to be routinely performed on extensive clinical material, technically simpler and more rapid assays are needed than those we have available so far.

Diagnosis.—The fact that neuroblastomas and melanomas have TAA cross-reacting within the respective groups indicates that useful information may be gained from searching for cytotoxic lymphocytes and for blocking and cytotoxic serum antibodies in patients in which these tumors have been suspected but are difficult to diagnose properly. For such an approach to have any wider practical importance, however rapid and simple assays are needed. At present, relatively quick techniques are being developed in order to diagnose colonic carcinomas (100), which exhibit carcinoembryonic antigen (CEA) in the sera of tumor-bearing patients. What practical value these techniques will have for the diagnosis of colon cancers is unknown. If they prove to be useful, analogous types of assays may become applicable for diagnosis of such tumors as neuroblastomas and melanomas.

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EPSTEIN-BARR VIRUS INFECTION IN RELATION TO INFECTIOUS MONONUCLEOSIS AND BURKITT'S LYMPHOMA[1]

7028

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Introduction

When the Epstein-Barr virus was first disclosed in cell lines derived from Burkitt's lymphoma, it seemed to be a herald discovery in delineating a viral etiology in cancer. As has occurred at other times in medical history, this was followed by a series of rapid and sequentially defervescent revelations, with EBV now being recognized as an ubiquitous herpesvirus agent of higher primates. This agent has, however, still unexplained and provocative relationships to Burkitt's lymphoma and nasopharyngeal carcinoma. A brief historical note is appropriate to introduce these developments.

Burkitt's lymphoma, the most prevalent neoplasm of children in central Africa and certain other areas of the world (1–3), has epidemiological features which suggested an infectious etiology to many observers (4); these features include geographic patterns and age, familial and temporal clustering. Certain clinical aspects of this tumor's behavior suggested that immunologic mechanisms might be involved in its pathogenesis. Extraordinary sensitivity to minimal or modest irradiation or chemotherapy (5), high "cure" rates, and a significant incidence of spontaneous long-term remissions have been cited in this context. Two key developments were responsible for putting this hypothesis to its first critical tests, namely, the establishment of permanent, stable suspension cultures of cells derived from such tumors—lymphoblast cell lines (6–10), and the observation by Klein et al (11) that sera from patients having this tumor contained high levels of antibody to a membrane antigen (MA) on living cells, both from fresh tumor and cell lines derived therefrom. These cell lines provided not only for Epstein's visualization of the herpeslike virus particle (6), now EBV, but also a source of infective virus and virus products for characterization and immunologic analysis.

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[1] Abbreviations used in this paper include: BL (Burkitt's lymphoma); EA (early antigen); EBV (Epstein-Barr virus); IM (infectious mononucleosis); LCL (lymphoblast cell line); MA (membrane antigen); MIF (membrane immunofluorescence); MLC (mixed lymphocyte culture); NPC (nasopharyngeal carcinoma); PA (precipitating antigen); VCA (virus capsid antigen); VNA (virus neutralizing antibody).

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Concomitantly, Henle and colleagues (12, 13) described a serum antibody, detected by either indirect fluorescence on acetone-fixed smears or complement fixation, which was directed toward a cytoplasmic antigen in cells of the LCL. They soon established that this antigen was a part of the virus particle itself, specifically its capsid protein (Virus Capsid Antigen). Antibody to VCA has since been found in the serum of a high proportion of the American adult population.

Apparently on the basis of chance observations when a previously serum-negative member of their laboratory staff became VCA-positive during the course of clinical infectious mononucleosis, the Henles provided the first evidence in 1968 (14) of the probable etiologic role of EBV in IM. Their discovery of the probable agent in this previously unexplained disease led to studies of cell lines derived from IM patients and to general interest in the significance of EBV infection of lymphoreticular cells. The discussion which follows will be focused upon (*a*) the role of EBV in infectious mononucleosis; (*b*) the role of EBV infection in BL and related tumors; (*c*) a description and critique of cellular and immunologic aspects of EBV infection; and (*d*) interpretations of available data in terms of the biologic significance of EBV infection.

ROLE OF EPSTEIN-BARR VIRUS IN INFECTIOUS MONONUCLEOSIS

Since its earliest comprehensive description (15, 16) IM has been strongly suspected of having a viral etiology, based upon clinical and epidemiologic patterns, organ systems involved, and heterophile antibody responses. The evidence to be described provides persuasive evidence that EBV is the causative agent.

Clinical and serologic evidence.—In patients having classical IM, accompanied by increased heterophile antibody titers (14, 17–23) (Smith, R. T. and Bausher, J. C., unpublished data), anti-VCA appears de novo in the serum within 5–7 days of the onset of clinical disease, with only rare exceptions. The titers rise initially, fall to lower levels, and remain positive indefinitely. Anti-MA, anti-EA, and neutralizing antibody (VNA) titers rise later and are more transient (see Table 1). A modest but undetermined proportion of patients showing some clinical manifestations of IM, with rising anti-VCA, fail to develop significant heterophile titer elevations (20, 22). On the other hand, elevated heterophile antibody titers are rare in VCA-negative patients. An unexplained antibody (24) present in the serum of most IM patients reacts with Newcastle disease virus but is apparently not related to heterophile antibody or other known viruses.

TABLE 1. Immunologic Responses to Various Antigenic Components of EBV or EBV-Infected Cells

Antigen or component	Immune responses reported		
	Burkitt's lymphoma	Infectious mononucleosis	Normal population
MA—membrane antigen complex associated with EBV infection of lymphoid cells; appears after EA but before VCA or viral DNA synthesis in actively infected cells in vitro.	Direct or indirect MIF antibody present in high titer in nearly 100% of patients during disease; decreases during remission; rises prior to recurrence; elevated consistently also in NPC.	Rise in titer follows anti-VCA and anti-EA; falls to low or undetectable levels after convalescence.	Present in serum of: >80% African adults <20% American adults <2% American children.
MA-1 (M) MA-2 (K) MA-1, 2	Antigenic subsites in MA complex on LCL detected by sera from different BL patients using the direct MIF test in a blocking technique.		
EA—cytoplasmic antigen appearing early after EBV infection of cells in vitro prior to DNA synthesis, MA, or VCA.	Elevated antibody titer present in most patients; persists throughout disease.	Antibody titers show transient early rise paralleling anti-VCA response.	Usually negative.
VCA—cytoplasmic antigen associated with EBV capsid; appears after viral DNA synthesis.	High titers of antibody detected by immunofluorescence or CF in nearly all patients; corresponding with high titer anti-MA and anti-EA; also very high in NPC, Hodgkin's disease, sarcoid, and leprosy.	Early rise of antibody to high titer; declines slowly and persists at lower levels indefinitely.	Absent or very low in American children; present in rising frequency during adolescence until 75–85% positive in adults; present in 90% of African children over 2 yrs of age.
PA—precipitating antigen is presumably virus particles and membrane components of established cell lines containing EBV.	Present in high titer in 50% BL patients, especially after recurrences; also high in NPC patients.		11% of healthy adults and those with unrelated diseases.
VNA—neutralizing capacity of sera to prevent in vitro infection of EBV-negative lymphoid cells by EBV particles; target is probably virus envelope antigen identical to MA complex.	High titer in most patients.	Rise early in course of disease, paralleling MA antibody levels.	Not present in sera which do not contain complement fixation or VCA fluorescent antibody.

Epidemiologic evidence.—Regardless of the route of spread, IM appears to be infectious. Repeated examination of sera from Yale students (17, 18, 25–27) showed that anti-VCA was present in a small proportion of entering students (~25 percent) and increased in frequency throughout the next 4–6 years to ~75 percent. More important, it was found that those positive on entry to Yale did not develop clinically evident IM during their four years;

all cases of IM occurred in those negative on admission. The conversion rate in this and other studies of young adults in the college age group is estimated to be 130–160 per 1000/yr. As judged by conversions from negative to positive sera during the same interval, this incidence of EBV infection is nearly double that of clinically evident IM.

Thus, anti-VCA was associated with resistance to IM and its absence with susceptibility. Additional indirect but correlative evidence comes from population studies which indicate that American infants and small children are generally seronegative for anti-VCA; the frequency of positives rises sharply during adolescence and young adult life corresponding to age-specific incidence of IM.

Evidence of direct transmission.—IM has long been suspected of being transmitted by intimate person-to-person contact; whether this hypothesis has merit or not has yet to be critically tested. Were this the case, intrafamilial spread would also be expected. No consistent sibling cluster patterning was uncovered, however, in retrospective examination for anti-VCA in family sera from the Cleveland study (28). Although direct person-to-person transmission under natural conditions is not known, well-documented transmission by inoculation comes from two sources. In a study of post-perfusion syndrome (29), examples of serologic conversion and concomitant clinical disease were recorded when VCA-positive blood was given to negative recipients during open-heart surgery. It should be noted, however, that cytomegalovirus, a related DNA virus, was found to be responsible for other examples of this syndrome. In addition, Grace has informally related (30) an instance of a serum-negative leukemia patient developing antibody and a suggestive clinical illness after receiving EBV-containing material, but this has not been repeated.

Direct evidence of virus presence in infectious mononucleosis.—No routine method of virus isolation and culture supports growth of EBV, and the agent has not been found in the usual secretions or body fluids. However, EBV is invariably present in lymphoblastoid cell lines isolated from patients who have the active disease or have recently recovered. In our laboratory all of over 60 such lines contained the VCA antigen. In contrast, no evidence of VCA has been found in the fresh peripheral leukocytes of patients from whom the cell lines were derived. Because anti-MA is present in the serum of such individuals, this result may inaccurately represent EB antigen expression prior to the appearance of serum antibody (31).

Thus, a variety of evidence favors an etiologic role for EBV in infectious mononucleosis, despite fragmentary evidence for direct transmission. Moreover, it appears that EBV infection is ubiquitous in man (and chimpanzees, 32), that most cases in young children are either asymptomatic or "atypical," and that at least half the infections of young adults are similarly asymptomatic or associated with mild illness. The pathogenesis of this complex, multisystem disorder remains to be unraveled.

ROLE OF EPSTEIN-BARR VIRUS IN BURKITT'S LYMPHOMA AND RELATED MALIGNANCIES

The discovery of EBV in relation to Burkitt's lymphoma initiated intensive efforts to define its role in this tumor. The most accepted working hypothesis holds that it is an oncogenic virus which rarely, under conditions yet to be defined, induces cellular transformation with subsequent proliferation. Another operational viewpoint, tentatively shared by the authors, is that all EBV infections are associated with oncogenic transformation at the cellular level and, as a corollary, that the usually benign outcome of these events is determined by effective host defense mechanisms. A third view holds that, as an ubiquitous virus of man, EBV is a fellow traveler in lymphoreticular tumor cells with a significance no different from its presence in the lymphocytes of IM patients and normal individuals. Some of the data upon which a decision between these different hypotheses, or other options, must be based will be reviewed briefly.

General biologic features of Burkitt's lymphoma.—This tumor is prevalent in central Africa and New Guinea in children between the ages of 3 and 13, rarely occurring in the first year of life or after age 16 (33). The number of authentic American patients is relatively small in contrast (34–36). Burkitt's lymphoma is peculiar among lymphomas in having an affinity for maxillar and mandibular areas and the endocrine organs; it is rarely found in the lymphoid tissues. Remissions induced by drug therapy or irradiation are often completely tumor-free.

Immunologic aspects.—Since Klein and colleagues (11) first described indirect membrane immunofluorescence when serum of BL patients was reacted with their own tumor cells, the techniques for detecting MA have been refined considerably; most critical recent information has been obtained by a modification involving blocking the direct MIF reaction (37–39). These studies have led to the conclusion that anti-MA is present in BL serum in high titer and that such antibody detects MA both on autochthonous and allogeneic Burkitt biopsy cells and on the various cell lines derived therefrom. This antibody, therefore, reacts with a shared antigen on tumor cells from all BL patients. The antigen is not present on bone marrow or peripheral blood cells, whether of autochthonous or allogeneic origin (40, 41). It is distinctive from HL-A and other leukocyte antigens (41). Anti-membrane antigen present in these patients is chiefly, if not exclusively, IgG class (42). Development of the direct MIF blocking technique permitted Klein and colleagues to detect two, and probably three, antigenic variants in the MA complex (43, 44). Each antigen of this complex is apparently present in all derived cell lines and located in geographically overlapping areas of the cell surface membrane. However, some BL patients' sera contain anti-MA-M, others anti-MA-K, and still others both. The MA complex appears to be related to EBV envelope antigens, since only MA-containing cells are able to absorb EBV-neutralizing activity of serum (44, 45). Membrane antigen present on cells in LCL appears identical to that on fresh biopsy cells, as indicated by mixed cell agglutination where anti-MA was present on the surface of the biopsy cells (46, 47).

Anti-early antigen and anti-VCA are also present in high titer in BL patients, measured either by immunofluorescence or complement fixation. Such titers are most often concordant with high anti-MA levels (48). Early antigen and VCA are antigenically distinctive; antibodies to these are not absorbed by MA-positive, VCA-negative cells (43, 49–51). Another antibody frequently present in BL serum is a precipitating antibody, described by Old and colleagues (52), which reacts with virus and/or virus-determined protein derived from an established cell line. Of interest was the observation that it reappeared in high titer in a patient during a fatal relapse after a long remission period during which it was not detected (53). The relationship of this antibody to the others has not been examined in detail. Lastly, an antibody is present in serum of BL patients and normal individuals which with complement is cytotoxic for lymphoblast line cells (54, 55), and, to a lesser extent, for normal lymphocytes. It apparently disappears from the serum during IM. This cytotoxic effect is heat-labile in contrast to the other antibodies described. Its significance with respect to EBV infection is uncertain.

In addition to antibodies stimulated in BL patients, the immunologic response to EBV or its cellular products includes delayed type hypersensitivity reactions directed toward antigens present in autochthonous tumor cells (56). It is of interest that 7 out of 12 patients skin-tested after tumor removal or during remission had positive tests and sustained remissions, whereas 4 out of 5 negative reactors had early recurrences. Reactivity was absent prior to tumor removal or chemotherapeutic induction of a remission.

Thus, the BL patient is evidently capable of marshaling all components of the immunological mechanism directed toward tumor MA. Antibodies are also directed toward viral components or virus-directed components which are not expressed in the tumor itself (EA, VCA) but in derived cell lines.

Epstein-Barr virus in Burkitt's lymphoma.—As in IM nearly every continuous lymphoblast cell line derived from BL tumor cells has contained EBV or its products (57). In contrast to the occurrence of MA in tumor cell membranes, no virus particles, VCA, or EA have been directly detected in fresh biopsy material or any other tissue of such patients. It is reasonable to conclude from the presence of MA on the tumor cell membrane that the tumor cell does contain EBV in some form.

Thus, patients having BL carry a tumor which expresses EBV-directed antigens. They develop an immune response of the delayed sensitivity type directed toward membrane components and antibodies which neutralize EBV and bind to both BL tumor cells and derived cell line membranes. This constitutes necessary but insufficient evidence that EBV is the oncogenic agent in BL.

Epstein-Barr virus in other disorders.—Similar high levels of antibody to EBV-directed antigens, including VCA, MA, and PA, have been found in two other malignant disorders, nasopharyngeal carcinoma (58–62) and Hodgkin's disease (63, 64), and in nonmalignant diseases including Boeck's sarcoid (65), acute lupus erythematosus (66), and leprosy (67).

Nasopharyngeal carcinoma is an undifferentiated cellular tumor giving evidence of EBV infection (68). In NPC, as in BL, concordantly high levels of anti-MA, VCA, and PA are found; in contrast, other cancers of the oropharynx and related tissues are not associated with such high levels. The possibility that this tumor has its origin in a thymus remnant has been raised both on histologic grounds (69) and because it may contain thymus-like antigens (70). If these data are correctly interpreted, NPC could represent a primary neoplasm of lymphoreticular tissues not unlike BL in its association with EBV.

In the case of Hodgkin's disease additional problems will make parallel observations difficult. Whereas NPC has cytologically and histologically distinctive features, the multicellular character in most cases of Hodgkin's disease makes it difficult to discern whether target cells derived for study arise from tumor or from normal cells which acquired EBV before Hodgkin's disease developed. It is of interest that impaired cell-mediated immune reactions (71) are a common feature in Hodgkin's disease, sarcoid, and related diseases.

CELLULAR ASPECTS OF EPSTEIN-BARR VIRUS INFECTION

The general nature of EBV-host interaction has been illuminated by examining immunological responses of individuals with EBV-associated diseases. On the other hand, it has been through examination of EBV infection at the cellular level that many critical issues of EBV infection have been defined and resolved; these will be discussed in terms of cell lines derived from various sources, some properties of these lines, their immunologic and oncologic significance, and their in vitro production by active virus infection.

Derivation of lymphoblastoid cell lines.—Suspension cultures of cells having lymphoblastoid morphology were originally derived from biopsy material from BL patients. It soon became evident that similar if not identical cell lines could be grown from peripheral blood leukocytes from IM patients. As more controls were sought, such lines were readily forthcoming from buffy coat cells of most normal adults. At one point major emphasis was given to serum hepatitis patients as a source of such lines (72, 73), and the need for phytohemagglutinin (PHA) or related mitogen stimulation (74) in producing cell lines from normal individuals was emphasized. It is now generally agreed that normal, anti-VCA-positive individuals are ready sources of LCL and that PHA activation is not required. Cell lines derived from these various sources have so many common features and so few significant differences, that they will be described as though identical; certain distinctions will then be cited.

The techniques currently employed in this laboratory for deriving the lines are simple and involve minimal "art." Leukocytes, usually 10^7 per culture, derived from whole blood by differential sedimentation, are put in 1 ml of RPMI-1640 culture medium containing 20 percent fetal calf serum or autochthonous donor serum and incubated at 37°C in a 5 percent CO_2 atm. Medium is added as needed, and after between 20 and 50 days, clumps of lymphoblastoid cells in suspension become evident and, with appropriate husbandry, evolve into continuous stable lines of lymphoblastoid cells. The line may be cloned to assure monoclonal characteristics at least temporarily (75, 76). Excluding laboratory accidents, this technique gives nearly 100 percent cell lines when the leukocytes are derived from IM patients and 50–60 percent when taken from normal young adults.

In our laboratory every one of over 60 lines derived contained EBV-associated antigen (VCA). Moreover, no cell line was derived from an individual who did not have anti-VCA in his serum. In many attempts in this and other laboratories, comparable cell lines have not been obtained from VCA-negative individuals.

Properties of cell lines.—Cells comprising these lines have the uniform morphologic appearance of lymphoblasts, although they vary considerably in details of cytoplasmic and nuclear organization with phase of the cell cycle and culture conditions. The cytoplasmic organization is that of a synthetically active, secreting cell, including multiple polyribosomes, some rough endoplasmic reticula, a highly developed Golgi area, and many mitochondria. Lipid-containing cytoplasmic inclusions are frequently present, as in BL tumor cells, but these are of unknown significance.

The cells divide rapidly as clusters in suspension, having a doubling time of 24–36 hr depending upon culture conditions. Cell division rates seem to have a reciprocal relationship both to production of EB virus particles and virus-associated antigens. Conditions which favor the latter—temperature of 32°C (77), low arginine content of medium (78, 79), or prolonged incubation (7–8 days in contrast to 2–3 days) without feeding—essentially stop cell replication. This is a property apparently shared by herpes simplex virus-infected cells (80).

Relation of cell lines to Epstein-Barr virus.—With few exceptions, every cell line examined in our laboratory and others has yielded visible evidence of herpes-like virus particles (by electron microscopy) and/or EBV-associated antigens (by immunofluorescence). The identity of the herpes-like particles with VCA antigens was established by the Henle group (81). Epstein-Barr virus particles are found in 0.001–0.1 percent of cells only, even in those lines which are excellent virus producers such as P3-J (Jijoye). Sampling problems, however, make direct electron microscopy techniques of limited value in determining virus presence or absence. Immunofluorescence detects EB virus or virus-associated antigens in a much higher proportion of cells grown under the conditions described above as favoring virus replication. Virus capsid antigen, detected in acetone-fixed, nonliving smears of cells, has a cytoplasmic and occasionally nuclear distribution and occurs in 0.1–10 percent of cells (82). Under optimal conditions MA complex is detected on up to 80 percent of living, unfixed cell membranes. Cytoplasmic or nuclear antigens are not seen in the latter technique because the reagents do not penetrate living cell surface membranes.

Modulation of the MA complex.—The MA complex cannot be considered stable even in cells having extensive MA representation on their surfaces. When anti-MA is reacted and binds with MA at the cell surface at 37°C, modulation occurs at a rapid rate (42). The MA-anti-MA complex disappears from the cell membrane within 30–60 minutes, and MA is not replaced. This modulation is prevented by incubating the cells at 4°C or in Na-azide. Evidence is accumulating that modulation is the result of internalization by some version of micropinocytosis. Modulation in this context may have more general significance in that it would represent a mechanism whereby antibody molecules could interact specifically with cell membrane components, the result being denudation of a cell surface of potential sites for recognition by cytotoxic cells in the sensitized host (83).

Products of lymphoblast cell lines.—Lymphoblast cell lines yield a variety of secretory products similar to those found in supernatants of lymphoblast cultures resulting from mitogenic stimulation of normal peripheral lymphocytes. Interferon (84–86), migration inhibitory factor (87), lymphotoxin (87), inhibitors of DNA synthesis (88), mitogenic factors (88), HL-A antigens in large quantity (89–91), and intact immunoglobulins (92–103) have been described in the culture fluids in which these cell lines are grown. The amount of immunoglobulin secreted by most cells is small, 15 mg per 10^9 cells per 24 hr in one study. Unequivocal evidence of both γ and μ chains and a single light chain in a single cell has been obtained in cloned lines (100, 102), though most lines contain either γ or μ and κ or λ light chains. Most have small amounts of γM immunoglobulin on their surface, but rare examples of cells having γG or large amounts of μ and κ chains on their surface—e.g., the Daudi line—have been found (104). Immunoglobulins are not present in all cells of a given line, rather in 5–40 percent, and probably depend upon the phase of the cell cycle (101).

Lymphoblasts from cell lines also appear to have complement receptors on their surface since they show immune adherence of sensitized sheep or human erythrocytes (Smith, R. T., unpublished data). Thus, immunoglobulin content and complement receptors would classify LCL cells as B cells if an analogy between human and mouse systems is appropriate.

Malignant properties of lymphoblast cell lines.—The question has frequently been raised whether cell lines derived in this way are "normal" or have malignant characteristics. By the criteria generally applied—uncontrolled proliferative behavior, lack of contact inhibition, and other nuclear and cytoplasmic structural characteristics used by cytologists—the cell lines resemble tumor cells. Three additional attributes appear to confirm their malignant potential: (*a*) virus-associated membrane neoantigens analogous to tumor-specific transplantation antigens, (*b*) chromosomal aberrations, and (*c*) lethal maligant tumor production when injected into immunosuppressed animals.

A central issue concerns whether or not MA endows the cell with receptors recognized by the original donor. In one-way mixed cell cultures (mixed lymphocyte culture) involving original donor leukocytes and either irradiated or mitomycin-blocked target cells from the autochthonous derived LCL, strong and consistent stimulation has been found in twenty donor-line pairs in our laboratory (Bausher, J. C. and Smith, R. T., unpublished data), and described in smaller groups by others (105–109). Compared to primary recognition of alloantigens in MLC, MA-containing cells stimulated 5–10 times as much ^{3}H-Tdr incorporation.

Self-recognition in this context was demonstrated regardless of whether the cell line was obtained from a normal individual or a patient having IM. High levels of stimulation were observed whether the combination was autochthonous or allogeneic, but in nearly every combination examined allogeneic pairs showed significantly greater stimulation than autochthonous pairs. This high level of autochthonous recognition is not due to changes in HL-A mosaic, for these are unchanged in LCL (90, 91). Stimulation does not appear due to absorption of culture medium ingredients by the cell lines. In this connection it is extremely important to be certain that mycoplasma have not infected the LCL, for these are potentially mitogenic and can give spurious stimulation (88). Experiments in progress in our laboratory indicate that the amount of MA in the cell lines is generally related to their capacity for stimulating autochthonous donors. Cells having high MA content, grown under conditions of arginine deficiency (79) and at 32°C, are more stimulatory than those grown under optimal conditions which give low MA content. This is consistent with self-recognition being based upon MA in the lymphoblast membrane.

Recognition antigens could arise from sources other than the virus genome, however. For example, these cells have PHA (110), concanavalin-A (Bausher, J. C. and Smith, R. T., unpublished data), and myxovirus receptors (Klein, P. A., Smith, R. T., and Bausher, J. C., unpublished data) on their surface in excess of normal peripheral leukocytes, and the density of their PHA receptors is increased by neuraminidase treatment (110). Time-anomalous differentiation antigens could be expressed in such cells and be responsible for self-recognition, as occurs in several neoplasms of man and animals. Thus, these cell lines appear to have one or more neoantigens which qualify them, by analogy, as tumor cells.

Chromosomal abnormalities have been found in a majority of cell lines examined. A relationship exists between culture age and the degree of aberration, since long-established lines have been found to have major chromosomal changes, and fresh ones from normal or IM patients are minimally aberrant (19, 103, 111). Controversy prevails as to whether an EBV-specific C-group marker exists; a secondary construction has been described near the end of the long arm of the #10 pair by several laboratories (112–116) but is thought to be nonspecific or insignificant by many (11, 116–121). Our own observations with freshly derived IM lines agree with the latter interpretation. Thus, chromosomal abnormalities are associated with fresh cell lines as is often but not invariably the case in malignant tumors, but these do not have a consistent pattern, which suggests that the EBV genome is located on or affects a specific chromosome.

Lastly, the ultimate test of malignancy is whether the cell or tissue will grow and kill an appropriate host. While it is not possible to test this property in syngeneic recipients, such cells have been infused in large numbers (122) without ill effects in one tumor patient and have not caused apparent tumors in allogeneic recipients. Of great interest are recent studies in which these cell lines, injected into immunosuppressed rats (123), mice (124), and hamsters (125) (newborn thymectomized and/or ALS-treated), grew rapidly as undifferentiated lymphomas which kill the host, were transferred serially, and, in some instances, became adapted to the point of not requiring immunosuppression.

It is difficult to escape the conclusion that these cell lines have the potential for both malignant behavior and strong antigenicity. If infection with EBV in vivo results in cells having comparable traits, the rarity of expression of oncogenic potential by such transformed cells could readily be explained by their strong antigenicity engaging an active and effective cell-mediated or cytotoxic antibody-associated immune mechanism. However, were autochthonous cell lines to be given to an immuno-suppressed or immunologically deficient host, as has been suggested as a potential means of immunologic engineering (126), this malignant potential might gain expression.

Active infection of cells by Epstein-Barr virus.—A major impediment to serious study of the mechanism of EBV infection has been the insusceptibility of any usual tissue culture cell lines or cell sources to support its multiplication; its range of acceptable milieu appears to be limited to the primate lymphoblast. Henle and colleagues (53, 114, 127) have developed a technique for infecting nonvirus-producing lines (46), or virgin normal lymphocytes taken from newborn or young infants, with EBV prepared from a high virus-yielding permanent cultured cell line. This has been widely confirmed (121, 128–131). Infection requires a high but undefined multiplicity of virus particles; this suggests that most particles are either incomplete or that the cells are relatively insusceptible to infection.

Using this technique of in vitro infection, the mechanism of virus infection has been somewhat clarified. Six to ten hours after infection of such cells, EA appears in the cytoplasm, followed by MA in the membrane at 10–13 hr. Neither of these steps is altered by inhibitors of DNA synthesis. Virus particles appear after 12–14 hr. Every cell does not go through this complete cycle of infection, however. Most infections were abortive, resulting only in EA or MA. A small but variable proportion produce VCA, and an even smaller proportion produce virus particles. Evidence that active virus infection is occurring in the susceptible cells, rather than this representing specific mitogenic stimulation followed by unmasking of latent virus, derives from the observation that serum from convalescent IM or BL patients neutralized EBV, preventing transformation and virus replication (120). In addition, infection was prevented by heating the virus at 56°C or by ultraviolet irradiation, which did not affect its antigenicity.

The technique of DNA hybridization has been applied recently by several investigators (132–134) to determine presence and degree of EBV DNA representation in the nuclei of various established lymphoblast cell lines or cells from BL and NPC. Significant homologies were detected between EBV and DNA from cell lines derived from normal individuals, IM patients, and BL patients. The number of genome equivalents of virus in the cell lines was estimated at 30–100 (134) by the sensitive method of DNAcRNA annealing, comprising up to 1.6 percent of the cell genome. Similar studies established that cell lines derived from BL which, by all immunologic methods, are free of any virus-related antigen (e.g., "Raji"), contain EBV DNA in their genome.

Most significant to the relationship between EBV and BL or NPC is the finding that every example of tumor cells examined contained EBV DNA in quantities of from 1 to 26 genome equivalents (132, 133). Moreover, the number of EBV genome equivalents per cell was constant in cells taken from multiple sites on a BL tumor, thus providing evidence for the clonal nature of these tumors (135). None of a variety of other neoplasms, including Hodgkins disease, contained EBV DNA. Control tumors included several from individuals having anti-MA, anti-EA, and/or anti-VCA in their serum at levels equivalent to BL or NPC patients. Hybridization attempts using herpes simplex virus DNA reveal no homologies in BL or NPC. Tumors taken from animals with Marek's disease, an oncogenic DNA herpesvirus tumor of chickens, also failed to show EBV homologies, although said to share a common antigen with EBV (136). Taken with immunologic evidence, these data provide a secure basis for concluding that BL represents an EVB-carrying tumor, even though it expresses only the MA complex. This is in contrast to LCL of tumor, IM, or normal origin which usually express VCA, EA, and MA in addition to containing infectious virus.

Mechanism of lymphoblast cell line proliferation.—In conclusion, the question of why EBV expression seems necessary to in vitro proliferation remains unanswered. In order to proliferate cells must come from individuals who have active or evidence of prior EBV infection, or they must be actively infected by virus if derived from an EBV-negative donor. Two general explanations of this requirement deserve consideration in future studies: (*a*) EBV, in directing expression of its antigens or virus production, impairs feedback mechanisms which normally control proliferation of lymphoid cells, and (*b*) EBV or EBV antigens initiate and then sustain ongoing proliferation by self-stimulation as, by analogy, in a breeder reactor. In the original peripheral leukocyte mixtures employed then, there would be either virgin immuno-competent cells or EBV-directed memory cells which are stimulated initially by EBV or EBV antigens. The resultant lymphoblasts would provide both the appropriate milieu for virus proliferation and the specifically reactive cells which can respond to ongoing antigen-driven proliferation. The second hypothesis seems susceptible to testing within the framework of current knowledge.

SYNTHESIS AND SPECULATION REGARDING THE SIGNIFICANCE OF HUMAN EPSTEIN-BARR VIRUS INFECTION

The evolution of information on EBV during the past five years has been rapid. Too many gaps remain, however, to develop a coherent picture of precise mechanisms in the pathogenesis of EBV-related diseases. The following statements seem sufficiently secure to guide future inquiry into less certain matters.

1. Epstein-Barr virus is a DNA virus of the herpesvirus group and is responsible for ubiquitous infections of man and certain primates regardless of geography. The route and means of acquisition of infection vary with age, geography, and other factors, but exact information is not yet available.
2. Infectious mononucleosis represents an unusual clinical expression of primary EBV infection in a limited age group. Most primary infections are either asymptomatic or not recognized as of EBV etiology.
3. After any primary EBV infection the virus becomes occult in some or all lymphoid cells of the intact normal individual. This state of latency is abrogated by in vitro culture conditions, resulting in establishment of LCL which express the virus genome in both antigenic products and infectious virus. It is probable that this occurs in vivo as well, but that has not been directly proven.
4. Host responses to EBV infection include antibodies to virus-directed MA, EA, and VCA. In addition, both in vitro and in vivo evidence exists for a strong, immune-type cellular recognition response directed toward the MA complex.
5. Burkitt's lymphoma and NPC are tumors specifically associated with exaggerated antibody responses to EBV; although neither tumor contains EBV particles, the EBV genome is present in both, in contrast to other tumors studied.

Several important questions remain unanswered, however, including (*a*) the mode of transmission of EBV infection, (*b*) the mechanism of maintenance of latency in normal individuals, and (*c*) whether EBV is actually an oncogenic agent in BL and NPC.

Answers to these questions depend upon resolving the more general problem of the nature of EBV infection. Although it seems very likely that it is acquired de novo as a primary postnatal infection, two other possibilities have been considered. For example, EBV infection could represent a vertically transmitted universal human viral agent which does not gain expression until some critical event permits its first emergence. No evidence for this exists, but the appropriate hybridization studies by sensitive techniques can establish the presence or absence of the latent EBV genome in some type of lymphoid cell of the fetus or infant. A related possibility is that EBV requires a helper or rescue virus in order to awaken from latency and become infectious and/or oncogenic, as is the case in certain avian leucoses (137). With one possible exception (138), this has no basis in results of in vitro studies to date.

Assuming person-to-person postnatal transmission, possible directions in which to look for the means of transmission have been delineated. That the agent is limited to intracellular growth in lymphoid cells suggests transmission via living lymphocytes. A direct mode of passage is quantitatively feasible as well, since at least one in $<10^6$ cells must carry the latent EBV genome; this is based on the fact that virus-containing cultures can be established from this few peripheral leukocytes.

The route of inoculation, however, remains obscure. Burkitt has repeatedly pointed out the correlation of malaria and BL in Africa, suggesting that EBV infection might be mosquito-borne (139). Inoculation of cells via secretions is also a possibility in this context (kissing and sexual intercourse would both fulfill this requirement), although the practical problems of obtaining direct epidemiologic evidence have been extremely difficult to solve in our own experience, and family patterns are not consistent with this route.

By what mechanism latency is maintained after initial infection is unknown. According to present knowledge neither recurrent infectious mononucleosis nor other manifestations of recrudescent EBV infection occurs in man, as is the case with herpes simplex and varicella. Therefore, it can be assumed that a highly effective mechanism exists which either prevents expression of the viral genome or destroys cells which escape such suppression. How the virus genome might be suppressed is unknown. Clear evidence of autochthonous cellular recognition of MA by lymphoid cells of EBV-positive individuals, however, provides a means for identification of cells escaping latency and their destruction. Neutralizing antibody in the circulation would probably prevent dissemination of infective virus particles. Unneutralized MA-EA, and VCA released in this process would act as stimuli for recall antibody synthesis, thereby explaining the continuing presence of anti-VCA after primary infection. Cell-to-cell transmission would enable emergent stem cells to acquire EBV and permit lengthy persistence of latent infection. In patients having deficiencies in cell-mediated immunity, including Hodgkin's disease, sarcoid, and some cases of leprosy, this postulated surveillance mechanism would probably be less effective and permit higher intensity antigenic stimulation and thereby high-level anti-EBV antibody. It might also be predicted that primary EBV infection in infants having congenital immunologic deficiency would either be severe or present unusual clinical manifestations.

Finally, the question of whether EBV is the oncogenic agent in BL and NPC requires consideration, even though an answer is not currently available. Both tumors contain the viral genome, and both stimulate intensive immune responses to EBV-direct antigens. Burkitt's lymphoma is a lymphoid tumor of cells similar in many respects to the lymphoblastoid cells which emerge in established cell lines from BL tumor, IM patients, and normal individuals. The origin of NPC is less clear but may represent a tumor originating in a thymic remnant. Here, similarities cease, however, for BL is an age-specific disease, and NPC occurs in older individuals and has different geographic and other characteristics.

A working hypothesis attempting to relate BL, IM, and EBV infection can be constructed, based upon analogy to virus-associated animal tumors such as Marek's disease. This idea holds that when EBV infection is acquired after a critical postnatal period of life, the result is infection, either with or without the clinical manifestations of IM, depending upon age-related variables. Such infections are conceived of as being oncogenic in that they give rise to virus-transformed lymphoid cells comparable to those emerging in vitro. However, cell-mediated surveillance mechanisms and, perhaps, cytotoxic antibody, stimulated as a result of infection, come into play and destroy those cells which constitute potential tumor clones before they become autonomous and insusceptible to immunologic attack. The infection then becomes latent by control of cell proliferation in the lymphoreticular system and remains controlled throughout life. If the primary infection is acquired during a critical postnatal period, this hypothesis holds that the response in most individuals would be as in older age groups. A small but significant proportion of such infections, however, would lead to uncontrolled proliferation of a clone of transformed cells, a nest which escapes surveillance mechanisms or stimulates a blocking antibody and, in time, becomes expressed as BL. A helper or rescue virus role in vivo is not excluded; it could be a direct one through concomitant infection or an indirect one by a temporary state of immunosuppression.

Regardless of whether this hypothesis ultimately proves to be correct in all assumptions, it is certain that further examination of EBV infection as a model for virus-associated and virus-targeted lymphoreticular system surveillance offers a potentially high yield of information of medical and general biologic interest.

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THE PRENATAL DIAGNOSIS OF INBORN ERRORS OF METABOLISM[1]

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The advent of prenatal diagnosis represents the most significant advance yet in the detection and prevention of irreparable mental defect and fatal genetic disease. This has occurred because of the many important advances in the technology of tissue culture and the biochemical methods applicable to it during the past two decades. Concomitantly, inherited metabolic disorders have been characterized in a stepwise fashion from study of the disorders in vivo, to the delineation of specific enzyme deficiencies in tissues, to the recognition of these deficiencies in cultured skin fibroblasts, leukocytes, and finally cultured amniotic fluid cells.

We have recently summarized in detail the current status of the art of prenatal diagnosis (1). In this paper we will examine subsequent progress in the antenatal detection of the inborn errors of metabolism and emphasize certain biochemical variables in tissue culture, newer methodologic approaches, recent therapeutic efforts in genetic disorders and the importance of heterozygote detection.

In Table 1 the inborn errors of metabolism, which may usefully be diagnosed in utero, are reviewed. Discussion in the text has been limited to those disorders in which recent advances have been of particular importance or interest, and those in which prenatal diagnosis seems likely in the near future.

DISORDERS OF LIPID METABOLISM

Among this group of diseases, six gangliosidoses are now clinically recognizable (Table 1). All these disorders are characterized by a progressive degenerative neurologic state with onset from birth to early childhood, fatal outcome, autosomal recessive inheritance, neuronal lipidosis secondary to storage of ganglioside G_{M1} or ganglioside G_{M2}, storage of structurally related glycolipids, polysaccharides, or glycoproteins, and the absence or severe deficiency of specific lysosomal hydrolases (2).

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TABLE 1. Inborn Errors of Metabolism That Can Possibly Be Diagnosed in Utero

Disorders	Deficient enzyme activity or other features in tissues or cultured fibroblasts	Prenatal diagnosis	Useful tissue for postnatal heterozygote detection	References
DISORDERS OF LIPID METABOLISM:				
Fabry's disease*	Ceramidetrihexoside galactosidase	Made	wbc, fibroblasts	22–24
Gaucher's disease	Glucocerebrosidase (β-glucosidase)	Possible	fibroblasts	118, 121–123
Generalized gangliosidosis (G_{M1} gangliosidosis Type I)	β-galactosidase B and C	Possible	wbc, fibroblasts	4, 15–17, 19
Juvenile G_{M1} gangliosidosis G_{M1} gangliosidosis Type 2)	β-galactosidase B and C	Possible	wbc, fibroblasts	4, 20, 21
Tay-Sachs disease (G_{M2} gangliosidosis (Type I)	Hexosaminidase A	Made	serum, wbc, fibroblasts	3–6
Sandhoff's disease (G_{M2} gangliosidosis Type 2)	Hexosaminidase A & B	Made	serum, wbc fibroblasts	4, 10, 126
Juvenile G_{M2} gangliosidosis (G_{M2} gangliosidosis Type 3)	Partial deficiency of Hexosaminidase A	Possible	serum, fibroblasts	2, 11–14, 41, 156
Krabbe's disease (globoid cell leukodystrophy)	Galactocerebroside β-galactosidase	Made	serum, wbc, fibroblasts	26–28
Metachromatic leukodystrophy	Arylsulfatase A (sulfatidase)	Made	wbc, fibroblasts	30–33
Niemann-Pick disease	Sphingomyelinase	Made	uncertain	123–125, 127
Refsum's disease	Phytanic acid α-hydroxylase	Possible	fibroblasts	128, 129
MUCOPOLYSACCHARIDOSES				
Hurler's syndrome	Intracellular mucopolysaccharide accumulation, abnormal $^{35}SO_4$ kinetics, metachromasia	Made	Uncertain	48–56, 68, 131
Hunter's syndrome		Made	Uncertain	
Sanfilippo syndrome		Possible	Uncertain	
Scheie's syndrome		Possible	Uncertain	
Maroteaux-Lamy syndrome		Possible	Uncertain	132
Morquio's syndrome	Metachromasia	Potentially possible	Uncertain	133, 134
AMINO ACID & RELATED DISORDERS				
Argininosuccinic aciduria	Argininosuccinase	Possible	rbc, fibroblasts	110, 135, 136
Citrullinemia	Argininosuccinic acid synthetase	Potentially possible	fibroblasts	137
Cystathionuria	Cystathionase	Possible	plasma, urine	138, 139
Cystinosis	Intracellular cystine accumulation	Possible	wbc, fibroblasts	70–74, 79
Histidinemia	Histidase	Possible	Uncertain	187
Homocystinuria	Cystathionine synthetase	Possible	Uncertain	109
Hyperammonemia (Type II)	Ornithine carbamyl transferase	Possible	Uncertain	140, 141
Hyperlysinemia	Lysine-ketoglutarate reductase	Potentially possible	Uncertain	142
Hypervalinemia	Valine transaminase	Potentially possible	fibroblasts	143, 144
Ketotic hyperglycinemia	Propionyl CoA carboxylase	Possible	wbc, fibroblasts	130, 145

TABLE 1. INBORN ERRORS OF METABOLISM THAT CAN POSSIBLY BE DIAGNOSED IN UTERO

Continued

Disorders	Deficient enzyme activity or other features in tissues or cultured fibroblasts	Prenatal diagnosis	Useful tissues for postnatal heterozygote detection	References
Maple-syrup-urine disease:				
a. Severe infantile	Branched-chain keto acid decarboxylase	Made	wbc, fibroblasts	146–150
b. Intermittent	As above	Possible	wbc, fibroblasts	
Methylmalonic Aciduria				
a. Unresponsive to vitamin B 12	Methylmalonic CoA isomerase	Possible	wbc, fibroblasts	151–155
b. Responsive to vitamin B 12	Methylmalonic CoA mutase	Possible	wbc, fibroblasts	
Ornithine-α-keto acid transaminase deficiency	Ornithine-α keto acid transaminase	Possible	Unknown	111
DISORDERS OF CARBOHYDRATE METABOLISM				
Fucosidosis	α-fucosidase	Possible	fibroblasts	157–159
Glycogen-storage disease (Type II)	α-1, 4-glucosidase	Made	wbc, fibroblasts	80, 81, 160–164
Glycogen-storage disease (Type III)	Amylo-1, 6-glucosidase	Possible	wbc, fibroblasts	165–168
Glycogen-storage disease (Type IV)	Branching enzyme	Possible	wbc, fibroblasts	169, 170
Galactosemia	Galactose-1-P uridyl transferase	Made	rbc, wbc, fibroblasts	108, 171, 172
Mannosidosis	α-mannosidase	Possible	fibroblasts	159, 173, 174
Pyruvate decarboxylase deficiency	Pyruvate decarboxylase	Potentially possible	wbc, fibroblasts	175, 176
Glucose-6-PO_4 dehydrogenase deficiency	G-6-PO_4 dehydrogenase	Possible	wbc, fibroblasts	177
MISCELLANEOUS DISORDERS				
Acatalasemia	Catalase	Potentially possible	wbc, fibroblasts	178, 179
Chediak-Higashi syndrome	Unknown	Potentially possible	fibroblasts	180
Congenital erythropoietic porphyria	Cosynthetase	Potentially possible	rbc, fibroblasts	181
I-cell disease	Reduced activity of multiple lysosomal enzymes in cultured fibroblasts	Potentially possible	Uncertain	82–86, 182
Lesch-Nyhan syndrome*	Hypoxanthine-guanine phosphoribosyltransferase	Made	rbc, wbc, fibroblasts	89–92, 94, 97
Lysosomal acid phosphatase deficiency	Lysosomal acid phosphatase	Made	wbc, fibroblasts	98, 99
Orotic aciduria	Orotidylic pyrophosphorylase & decarboxylase	Potentially possible	rbc, wbc, fibroblasts	183, 184
Xeroderma pigmentosum	DNA "repair enzyme"	Possible	Unknown	185, 186

All disorders listed are autosomal recessive, except those marked * which are sex-linked. wbc = leukocytes, rbc = red blood cells.

Tay-Sachs disease (G_{M2} gangliosidosis Type I).—Deficiency of hexosaminidase A, recognized as the specific enzymatic defect (3), has been demonstrated in multiple tissues including cultured skin fibroblasts and cultured and uncultured amniotid fluid cells. Deficiency of hexosaminidase A in cultured amniotic fluid cells is being recognized increasingly in early pregnancy (4, 5).

Since hexosaminidase A and B are present in normal human serum (3), the opportunity to detect homozygotes and heterozygotes by serum hexosaminidase assay has become a reality. O'Brien et al (6), using a fluorometric assay for the estimation of both hexosaminidases in human serum, have clearly demonstrated the facility with which this technique can be applied clinically. Their assay exploits the different thermal stabilities of hexosaminidase A (heat-labile) and hexosaminidase B (heat-stable) (7). Total hexosaminidase is estimated in serum before heating and the activity of hexosaminidase A which is inactivated after heating is calculated by the difference. Overlapping values between heterozygotes and normal controls have not been troublesome, although the gap between the two groups seems narrow (6). This important contribution by O'Brien and his team has opened the way to mass screening procedures for serum hexosaminidase A activity in high risk population groups, for example Ashkenazi Jews. It is important to bear in mind O'Brien's observation that serum hexosaminidase A activity deviates appreciably from the range established for healthy controls in patients with diabetes mellitus, in those with debilitating illnesses which involve tissue necrosis, pregnant women, and babies younger than two months of age. Apparently heterozygote detection in these instances is not possible using the control values available (6). Kaback, however, has some observations which indicate that leukocytes instead of serum could be used in determining the heterozygous state in the second trimester (8). Suzuki et al (9) have drawn attention to the more clear-cut separation obtained between homozygote and heterozygote when results were expressed as the actual amount of substrate cleaved, rather than as a percentage of the total enzyme activity.

Cultured and uncultured amniotic fluid cells appear to be the most reliable samples for assay since they reflect the greatest difference between normals and homozygotes. Amniotic fluid itself appears less useful because of the smaller differential between the values of normals and affected fetuses (4). Hexosaminidase assays of amniotic fluid or uncultured amniotic fluid cells may be unreliable if contamination with maternal blood occurs (4). Furthermore, assays of cultured amniotic fluid cells may be unreliable if bacterial contamination occurs since some bacteria contain a heat-labile β-D-N-acetylglucosaminidase with an acid pH optimum and an electrophoretic migration very similar to hexosaminidase A (4).

Sandhoff's disease.—Two biochemical criteria unequivocally distinguish this disease from classical Tay-Sachs disease: the additional accumulation of globoside in visceral organs (10) and the total deficiency of hexosaminidase A and B. Because of the phenotypic similarity of these two diseases, enzyme assay is presently the only reliable means of differentiating between them. The importance of distinguishing between these two diseases is self-evident when heterozygote detection, counseling, and the prenatal monitoring of subsequent pregnancies is planned.

Juvenile G_{M2} gangliosidosis.—At least five affected patients have been reported (2, 11–13). Cerebral levels of ganglioside G_{M2} are 40–90 times normal in this disorder (13), which is a smaller accumulation than in Tay-Sachs or Sandhoff's diseases. O'Brien et al (14) have found a partial deficiency of hexosaminidase A in two patients with juvenile G_{M2} gangliosidosis. The deficiency has been demonstrated in brain and liver in one child and in serum and cultured skin fibroblasts in another. In cultured fibroblasts the deficiency of hexosaminidase A averages 25 percent of normal, whereas in serum the deficiency of hexosaminidase A is almost as severe as that seen in Tay-Sachs disease. Serum hexosaminidase assay is a simple way to confirm the diagnosis and to detect the heterozygous state.

Generalized gangliosidosis.—It appears that the accumulation of G_{M1} ganglioside in this disease is due to a profound deficiency of β-galactosidase which normally participates in its breakdown (15). Ho & O'Brien (16) have separated liver β-galactosidases into three components by starch gel electrophoresis, a fast-moving component (A) and two slower moving ones (B and C). In generalized gangliosidosis, all three components are markedly deficient. β-galactosidase deficiency is evident in leukocytes (17), urine (18), and cultured skin fibroblasts (19), as well as amniotic fluid cells (4). This enzyme assay can therefore be used to diagnose the homozygous state both pre- and postnatally, and to detect individuals heterozygous for this disease. Sloan and Fredrickson (as quoted) (4) have monitored a pregnancy at risk for generalized gangliosidosis. Enzyme assay indicated that the fetus was unaffected, and examination of the child at eight months of age revealed no symptoms or signs of the disease.

Juvenile G_{M1} gangliosidosis.—A profound deficiency of β-galactosidase has been demonstrated in tissues (20), peripheral leukocytes (4, 21), and in cultured skin fibroblasts (20). The deficiency of β-galactosidase in the liver is not as marked as that in generalized gangliosidosis. In contrast to generalized gangliosidosis, in one patient with juvenile G_{M1} gangliosidosis studied by O'Brien (4), β-galactosidase A was present at nearly normal levels with a deficiency of components B and C. Additional studies are required to determine the constancy of this finding. Nonetheless, it appears that the diagnoses of homozygotes prenatally and postnatally and the detection of individuals heterozygous for the gene for juvenile G_{M1} gangliosidosis are now possible.

FABRY'S DISEASE

Kint demonstrated a deficiency of α-galactosidase activity in leukocytes obtained from patients with Fabry's disease (22). Following these observations, others reported detection of the hemizygous patients and heterozygous female carriers by testing extracts of skin fibroblast cultures for α-galactosidase activity (23, 24). Based upon this information, Brady et al (24) studied a twenty-two year old female in the 17th week of gestation who had been identified as a heterozygous carrier through assays performed on extracts of her cultured skin fibroblasts. Assays performed on extracts of an acetone powder of the cultured amniotic fluid cells contained 1/10 of the α-galactosidase activity of preparations from control cell cultures. While these workers were unable to confirm the diagnosis of Fabry's disease by direct assays of α-galactosidase activity, their impression that the fetus had Fabry's disease was confirmed by direct analysis of the ceramide trihexoside in fetal tissue.

Addition of ceramide trihexosidase to the plasma of patients with Fabry's disease might well decrease plasma ceramide trihexoside and the rate of accumulation of glycosphingolipid in the tissues. Since the pure enzyme is not available, attempts have been made to infuse two patients with normal plasma (25). These workers showed that the amount of ceramide trihexoside in the plasma gradually decreased to about 50 percent by the tenth day after infusion, and they raised the possibility that periodic replacement of ceramide trihexosidase activity in the plasma of patients with Fabry's disease might decrease its rate of accumulation in tissues (25).

KRABBE'S GLOBOID CELL LEUKODYSTROPHY

This fatal recessive neurological disorder of infants appears to be due to deficient activity of galactocerebroside β-galactosidase (26). Demonstration of this requires the use of the specific substrate, galactocerebroside, since assays done with unnatural synthestic substrates, such as 4-methylumbelliferyl β-galactoside, do not distinguish homozygotes from heterozygotes. Besides various whole tissues, Suzuki & Suzuki (27) have demonstrated the activity of galactocerebrosidase to be extremely low in serum, leukocytes, and cultured fibroblasts from patients with Krabbe's disease. The heterozygous state can now be diagnosed from studies of serum alone. Not unexpectedly, galactocerebrosidase activity has also been found in cultured amniotic fluid cells which made it possible to diagnose an affected fetus in a mother at risk (28).

METACHROMATIC LEUKODYSTROPHY (MLD)

Metachromatic leukodystrophy has been reviewed recently in detail by Moser (29). Late infantile, juvenile, adult, and variant forms of this disease have been recognized through their different ages of onset and variable evolution. The disease is characterized by a deficient activity of arylsulfatase

A and by an accumulation of cerebroside sulfate. The activity of this enzyme is reduced in leukocytes (30), cultured skin fibroblasts (31), and cultured amniotic fluid cells (32). Heterozygote detection is now possible through arylsulfatase A assay of leukocytes (33) or cultured skin fibroblasts (32).

Of particular importance to the understanding of storage diseases in general are the recent observations by Stumpf & Austin (34). They have reported both qualitative and quantitative differences in arylsulfatase A activity in the various forms of MLD. In the light of their findings, efforts at making the prenatal diagnosis of the late infantile, juvenile, or adult forms of this disease will require the most careful evaluation and interpretation.

Metachromatically staining inclusions are not normally found in cultured fibroblasts in patients with MLD unless sulfatides are provided in the culture medium (35). Porter et al (36) have demonstrated that skin fibroblasts cultured from patients with the late infantile form of MLD incorporate arylsulfatase A from the growth medium. Inclusion granules formed in MLD fibroblasts upon exposure to sulfatides were cleared by subsequent supplementation of the growth medium with arylsulfatase A. Wiesmann et al (37) have reached the same conclusions in similar experiments. The demonstration by these workers of the temporary correction of the enzyme deficiency in cultured skin fibroblasts has obvious therapeutic implications. In this regard Greene et al (38) reported the treatment of a patient with intravenous infusions of arylsulfatase A. They noted an increase of the enzyme activity in liver but not in brain and no clinical change.

LACTOSYL CERAMIDOSIS

In 1970, Dawson & Stein (39) described this disorder which is characterized by neurologic impairment, cerebellar ataxia, spasticity, hepatosplenomegaly, foam cells in the bone marrow, and high concentrations of lactosyl ceramide in blood, bone marrow, urine sediment, liver, and brain. A specific deficiency of lactosyl ceramide galactosidase was found and subsequently confirmed in cultured skin fibroblasts. Using the latter tissue, Dawson (40) has also been able to identify the heterozygous state. Undoubtedly, activity of this enzyme will be detected in cultured amniotic fluid cells, thereby making a prenatal diagnosis of lactosyl ceramidosis possible.

WOLMAN'S DISEASE

Wolman's disease is a rare lipidosis manifest in early infancy as failure to thrive with steatorrhea, hepatosplenomegaly, and adrenal calcification (42). There appears to be variation in the clinical expression of this disease from the acute infantile form with inevitable early death to disorders of later onset which follow a less severe and more prolonged course (43). Despite variation in clinical expression, the different forms of the disease appear to be characterized by the same enzymic abnormality, deficiency of an acid lipase acting on triglycerides and chloesterol esters (43). Massive accumulations of triglycerides as well as free and esterified cholesterol occur in the liver and spleen in these patients. Silverberg et al (44) have suggested that the defect in this disease is in the degradation of both triglycerides and cholesterol esters. Lake & Patrick (45) have demonstrated that lipid storage in the affected tissues is confined to intracellular bodies possessing histochemical and ultrastructural characteristics of lysosomes.

Cultured fibroblasts from two patients with Wolman's disease have been shown to contain elevated amounts of cholesterol, and in one case to incorporate an increased amount of ^{14}C mevalonate into cholesterol (46). Preliminary studies by these latter workers indicate that acid lipase deficiency is indeed demonstrable in cultured fibroblasts from a patient with this disease. Since leukocytes as well as liver and spleen from patients with this disease possess greatly reduced activity of acid lipase, a useful method is now feasible for the diagnosis of clinical variants of Wolman's disease. At the same time, the differentiation of heterozygotes becomes a possibility (47). As yet no one has studied acid lipase activity in cultured amniotic fluid cells.

THE MUCOPOLYSACCHARIDOSES

In the original studies of Danes and Bearn and others (48–50), cultured skin fibroblasts from patients with either the Hurler (Type I) or Hunter (Type II) syndromes were shown to accumulate mucopolysaccharides. Most of this appears to be dermatan sulfate. Heparin sulfate is found in excess in the patient but has not yet been shown to be formed by fibroblasts in culture. In the kinetic experiments of Fratantoni et al (51), skin fibroblasts from patients with the Hurler or Hunter syndrome and subsequently cultured amniotic fluid cells from an affected fetus showed excessive accumulation of radioactive mucopolyasccharide (52). In classic experiments, Fratantoni et al (53) demonstrated that when fibroblasts from a patient with Hurler's syndrome are cultured with fibroblasts from a patient with Hunter's syndrome, each appears to cancel out the other defect so that excess mucopolysaccharide is no longer produced. It appears that fibroblasts of one genotype can supply a factor that cells of the second genotype lack. In further studies these workers showed that the ability to correct the defect of Hurler or Hunter cells appeared to be a property of all other fibroblasts (54). They have been able to separate the Hurler, Hunter, and Sanfilippo (Type III) genotypes (54, 55). While in other studies Wiesmann & Neufeld (56) have demonstrated that cultured fibroblasts from the Scheie and Hurler syndromes are deficient in the same factor.

Efforts have been made to purify and characterize the corrective factors. They appear to be heat-labile macromolecules that behave as proteins (54, 55) which are found both inside cells and in the culture medium. From its electrophoretic mobility, the "Hunter factor" has been estimated to have a molecular weight of 65,000 (57). From a clinical viewpoint, these factors (if present in serum at all) probably do not cross the placenta, since in vivo maternal-fetal "correction" is not apparent. Detection of the absence of these factors in amniotic fluid or cells may someday provide more clear-cut prenatal diagnosis of the mucopolysaccharidoses than that now available.

A variable deficiency in β-galactosidase has been noted in liver, brain, and skin of patients with the Hurler and Hunter syndromes (55, 58, 59). However, in skin fibroblasts cultured from patients with Types I, II, and III mucopolysaccharidoses, only about a 50 percent reduction in the mean β-galactosidase activity was detected (60). In addition, the electrophoretic pattern of the β-galactosidase activity in these fibroblasts showed the presence of all the forms of the enzyme seen in normal cells. Benson and coworkers (61) reported essentially the same conclusions from their studies of Types I and III mucopolysaccharidoses. In confirming the β-galactosidase deficiency in liver in Type I, Patel et al (62) noted also deficiency of hyaluronidase in this organ. Hyaluronidase deficiency has not yet been demonstrated in cultured fibroblasts in Type I disease, but dermatan sulfate and hyaluronic acid do accumulate in these cells (63). An alternate explanation to β-galactosidase deficiency for the accumulated mucopolysaccharides in affected fibroblasts may relate to hyaluronidase and sulfatase deficiencies in these cells (62).

The most prominent storage compound in tissues of patients with mucopolysaccharidoses are the L-iduronic acid-containing polysaccharides, dermatan sulfate and heparan sulfate. Dermatan sulfate is partially degraded by hyaluronidase (64) the activity of which could nevertheless not be demonstrated in normal or Hurler-cultured fibroblasts (65). The complete degradation of dermatan sulfate seems to require the stepwise action of appropriate sulfatase, β-N-acetylhexosaminidase, β-glucuronidase, L-iduronidase, β-galactosidase, and β-xylosidase (65). All of these enzyme activities have been shown in tissues or fibroblast cultures, with the exception of L-iduronidase (58, 65). Recently, however, Matalon and associates (65) have found diminished L-iduronidase activity in cultured skin fibroblasts from patients with the Hurler syndrome and have raised the possibility that absent or diminished L-iduronidase activity may play a role in the pathogenesis of the mucopolysaccharidoses.

Hors-Cayla and associates (66) reported that the intracellular accumulation of mucopolysaccharides in affected fibroblasts disappeared when fetal calf serum was replaced by human serum in the culture medium. Based on these observations, Di Ferrante and associates (67) administered normal human plasma to patients affected by mucopolysaccharidoses I and II. The infusion was followed by decreased urinary excretion of large molecular weight mucopolysaccharides and increased excretion of mucopolysaccharide degradation products. These results indicate that normal human plasma may contain factors active in the normal degradation of dermatan sulfate and heparan sulfate which are absent in these disorders. The relation of these newest factors to those above is not yet clear.

These recent efforts represent a breakthrough in the treatment of the mucopolysaccharidoses. The preliminary nature of the observations and the need to assess the efficacy and safety of this approach is emphasized by these workers. It would therefore seem quite premature to influence unduly parents considering abortion of an affected fetus with information about the development of therapy at this early stage.

Heterozygote detection in the mucopolysaccharidoses remains uncertain. Those who may possibly be heterozygotes could best be counseled by advising amniocentesis for prenatal diagnostic studies to detect an affected fetus.

AMINO ACID AND RELATED DISORDERS

Clinically many of these disorders suggest themselves in the neonate through periodic vomiting, lethargy, seizures, ataxia, coma, mental retardation, or death. The clinical signs, especially in the congenital disorders of ammonia detoxication, are usually aggravated by a protein load and may quickly subside on a protein-free diet.

Daum et al (69) have recognized a new disorder of isoleucine catabolism involving the enzyme which catalyzes the formation of propionate from α-methylacetoacetate. This compound and its precursor, α-methyl-β-hydroxybutyrate are not normally present in significant amounts in body fluids, but were found in excess in the urine of a male patient who experienced three episodes of transient but profound metabolic acidosis. The concentration of these compounds increased greatly during an acute febrile illness and specifically after L-isoleucine loading. The parents of the child and one of two siblings of the patient were found to excrete constantly modest amounts of the two organic acids. In vitro oxidation of isoleucine-u-^{14}C by cultured skin fibroblasts was 40 percent of normal in the patient. It seems likely that this disorder will eventually be shown to be manifest in cultured amniotic fluid cells as well.

Nephropathic ("infantile") cystinosis, the lethal form of cystine storage disease, is characterized by the accumulation of crystalline cystine in multiple tissues including cultured skin fibroblasts (70–72). Both the benign "adult" form and the newly described adolescent form of this disease also accumulate intracellular cystine abnormally (73, 74). Recent observations suggest that normal lysosomes may have a mechanism for lysosomal transport or metabolism of disulfides with cysteine moieties, and that a congenital deficiency of this mechanism may be the distinctive molecular lesion in cystinosis (75).

Schneider et al (72) demonstrated a 100-fold increase in the content of free cystine in fibroblasts cultured from the skin of cystinotic children when compared to normal children, and a lesser increase (about 6-fold) in most individuals heterozygous for cystinosis. The concentration of free cystine in cultured skin fibroblasts in "adolescent" cystinosis is somewhat greater than in "adult" cells (73). The parents of these adolescents are clinically normal but retain free cystine abnormally in their fibroblasts to about the same extent as that found in heterozygotes for the "infantile" type.

Based on this knowledge, a new method has been developed for the study of small numbers of cystinotic cells in vitro (76). By exposing amniotic fluid cells to a pulse of radioactive cystine and studying the pattern of labeling of intracellular cystine, it was possible to diagnose the heterozygous state of nephropathic cystinosis in utero. Recent developments have advanced the understanding of cellular cystine flux (77) in and out of cells and have included the use of dithiothreitol to remove intracellular cystine (73, 78).

DISORDERS OF CARBOHYDRATE METABOLISM

Pompe's disease.—Recent studies indicate that the α-glucosidase activity present in cultured fibroblasts and amniotic fluid cells (but absent in Pompe's disease) have identical properties which are distinctly different from the α-glucosidase, in kidney tissue and amniotic fluid itself (80). Furthermore, the α-glucosidase in amniotic fluid appears to differ in heat stability from that found in kidney tissue. These studies failed to identify the origin of the α-glucosidase in amniotic fluid, but they do make clear that the prenatal diagnosis of Pompe's disease should be based on the demonstration of the α-glucosidase deficiency in the amniotic fluid cells rather than the fluid.

As another approach to the prenatal diagnosis of Pompe's disease, Hug et al (81) have done electron microscopy on 14 specimens of uncultured amniotic fluid cells. Ten of the samples served as normal controls, while four were from women who had had previous children with Pompe's disease. Uncultured amniotic fluid cells of two of the four high risk pregnancies contained glycogen accumulations within lysosomes, a finding regarded as the hallmark of this disease. The prenatal diagnosis was confirmed subsequently in both instances. Thus, direct electron microscopy of uncultured amniotic fluid cells can provide an additional method of prenatal diagnosis for Pompe's disease, a clear advantage of which is that it is possible within three days of amniocentesis.

MISCELLANEOUS DISORDERS

I-cell disease.—This disorder was first recognized as a result of studies on fibroblast cultures from patients (82, 83) and was given its name because these cells had many peculiar cytoplasmic inclusions now recognized to be not necessarily pathognomonic. This disorder is autosomal recessive and characterized by severe psychomotor retardation, short stature, frequent respiratory infections, excessive gum hypertrophy, and progressive Hurler-like changes of facial and body configuration, but no mucopolysacchariduria. Many abnormalities of lysosomal hydrolases have been noted in fibroblasts cultured from these patients (84–86). β-D-galactosidase and α-D-fucosidase were almost completely inactive; β-D-glucuronidase, N-acetyl-β-D-glucosaminidase, α-D-mannosidase, and arylsulfatase A were decreased to about 25 percent of control values. β-D-glucosidase, β-D-xylosidase, and acid phosphatase were slightly increased. Since most of these hydrolases are active in cultured amniotic cells, it may be possible to diagnose I-cell disease in utero.

The reason for these multiple enzyme deficiencies is not clear. Wiesmann et al (85) have presented evidence that the deficiencies may be due to increased leakage of lysosomal enzymes from the patient's fibroblasts, perhaps because of a basic defect in the lysosomal membranes. The patient described by Lightbody et al (84) had various low or nondetectable lysosomal enzyme activities in fibroblasts, whereas the enzyme activities were either normal or only partially reduced in the culture medium. It appears likely therefore that the lysosomal hydrolase deficiencies may well be secondary to another genetic defect.

Lesch-Nyhan syndrome.—The many aspects of this rare but well-known syndrome have recently been summarized (87, 88). Hypoxanthine guanine phosphoribosyltransferase was shown to be the enzyme deficient in this disorder by Seegmiller et al (89). This deficiency was demonstrated for cultured skin fibroblasts (90) as well as red blood cells (89), and later for cultured amniotic cells, followed by the detection of affected fetuses with this syndrome in utero (91–93). Red blood cells can be used to diagnose the hemizygous affected male but not the heterozygous female (94). Heterozygotes were first detected by Rosenbloom et al, using autoradiographic methods to demonstrate that some fibroblasts cultured from heterozygous females incorporated radioactive hypoxanthine into nucleic acids while other cells in the same culture failed to do so (95). Because of the various problems with the autoradiographic method, simpler techniques have been proposed based on the ability of purine analogues of hypoxanthine or guanine to inhibit the growth of cells having the enzyme but not that of enzyme-deficient cells (96, 97).

Lysosomal acid phosphatase deficiency.—This disorder described by Nadler & Egan (98) is characterized by vomiting, lethargy, opisthotonus, bleeding, and death in early infancy. Acid phosphatase activity in fibroblasts from a patient with this disease was decreased to 30 percent of normal in the total homogenates and to less than 2 percent in the lysosomal fraction. In another patient, the activity was reduced to 2 percent of normal in the total homogenate and not detectable in the lysosomal fraction (99), indicating genetic heterogeneity for this disorder. Furthermore, prednisolone induced phosphatase activity to 50 percent of normal in the partially deficient cells, suggesting that these patients might benefit from therapy. No effect was noted in the cells totally deficient in phosphatase (99).

Nonspherocytic hemolytic anemia.—Hereditary deficiency of erythrocyte phosphohexose-isomerase was shown by Baughan et al (100) to cause nonspherocytic hemolytic anemia. Krone et al (101) have reported that this deficiency is expressed in fibroblasts cultured from patients with this disorder. While the presence of the defective enzyme in cells derived from heterozygotes was revealed by an intermediate average specific activity, the wide range of PHI-activities observed in those cells studied precluded reliable heterozygote detection. Heat inactivation studies performed with sonicates of fibroblasts showed that exposure to 45°C for 60 minutes reduced the enzyme activity by 10 percent in the normal control, whereas heterozygous and homozygous individuals showed activities diminished by about 40 and 80 percent, respectively. Possibly the thermolability of the defective enzyme might provide the basis for a selective system to separate out heterozygotes from homozygotes.

Glycolytic enzymes and those involved in the hexose-phosphate shunt are important in maintaining the functional stability of erythrocytes and therefore it can be expected that additional hereditary enzyme deficiencies in these pathways will be recognized and allow the clearer definition of the hemolytic anemias. Opportunities for prenatal diagnosis of these disorders of red blood cell metabolism are then sure to follow.

Sickle cell anemia.—In elegant studies Hollenberg et al (102) have studied the incorporation of radioactive amino acids into hemoglobin A by reticulocytes of the second trimester fetus. They were able to demonstrate the synthesis of hemoglobin A in as little as 10 microliters of fetal blood despite the preponderance of hemoglobin F synthesis at this time in development. Substitutions in the beta chain are responsible for the majority of the hemoglobinopathies found in man. Therefore, knowledge of beta chain production in early fetal life is crucial if any of these disorders are to be detected prenatally. If a simple and safe method can be devised to obtain tiny samples of fetal blood in early pregnancy, then these or other techniques can be applied to the antenatal detection of sickle cell anemia and the other hemoglobinopathies.

Testicular feminization.—Whether testicular feminization is X-linked or a sex-limited autosomal dominant disorder remains uncertain. Recently cultured skin fibroblasts and amniotic fluid cells have been shown to metabolize testosterone (103, 104). Skin fibroblast cultures obtained from adult males and females showed a similar pattern of metabolism, whereas in children male cells were more active than female cells (103). New et al (103) found that fibroblasts from children with testicular feminization produced low amounts of the 5-α-reduced metabolites (5.3 percent) compared to control cells (80 percent). These observations suggested that testosterone 5-α-reductase might be decreased in the affected cells. Pinsky et al (104) showed that differences in testosterone metabolism between normal sexual and nonsexual skin persisted in cultured fibroblasts and agreed that testicular feminization was detectable in cultured fibroblasts. Amniotic fluid cells cultured from male fetuses with gestational ages of 15 to 17 weeks have been found to metabolize testosterone-like fibroblasts from young children (103), so that the stage is set for the prenatal diagnosis of testicular feminization, perhaps as the first of many genetic endocrine disorders in which a precise enzymatic deficiency will be defined.

BIOLOGIC AND BIOCHEMICAL CONSIDERATIONS

A complex set of variables affect human fibroblasts and amniotic fluid cells growing in vitro. Some of the more important of these have been recognized and briefly discussed as they may affect the prenatal diagnosis of the inborn errors of metabolism (1). They include cell generation time (105), degree of cell confluency (106, 107), importance of position in the culture growth cycle (i.e., log phase as opposed to stationary phase) (108), enzyme changes during gestation (33), differences between the lysosomal hydrolase activities of amniotic fluid cells and skin fibroblasts (109–111), and senescence in culture (112). For example, the concentrations of certain glycolipids increase 1- to 2-fold as the culture becomes confluent; and in addition these glycolipids gradually decrease in concentration on successive passages (113). Hexosaminidase activity has also been shown to increase late in the culture cycle (114). This effect of confluency is especially important when enzyme-specific activity is used for prenatal diagnosis. Clearly, control cells should be at the same stage of the culture cycle as the sample being studied.

At present there is much interest in the slowing of growth in confluent cultures and especially the roles of cell contact and changes in the medium (112, 115, 116). Also Ceccarini & Eagle (117) have drawn attention to the importance of pH as a determinant of cellular growth and contact inhibition. They demonstrated that both the growth rate and maximum population density of several normal fibroblast cell lines were maximal between pH 7.5 to 7.8. At these pH optima, cells attained densities 2 to 4 times greater than under usual culture conditions. At pH 8.3, they observed unbalanced growth in that protein synthesis continued in the almost complete absence of cell division.

The culture medium and the serum used present other important variables in assessing cellular enzyme activities. Beutler et al showed that β-glucosidase activity of fibroblasts and amniotic fluid cells grown in one medium was considerably higher than that grown in another (118). Thus, when a normal fibroblast culture was divided and subcultured both in MEM medium and in F-12 medium (very similar to F-10), the β-glucosidase activity at pH 4 in the former was found to be 2.7 times that of the activity of the latter. Differences in enzyme activity may also be due to differences in concentrations of fetal calf serum in the culture medium. Variations in the same assay (for β-glucosidase) have been noted in two different laboratories on fibroblasts cultured from the same patients with Gaucher's disease (118). The differences appear to be sufficiently great that what one lab will diagnose as a homozygote, the other may well call a heterozygote or even a normal!

The toxic effects of trypsin on cell cultures should be borne in mind. This enzyme not only detaches cells bound to charged surfaces but, on prolonged contact in simple salt solutions, it also has a profound effect on the physiological state of the cell. Proteases, which are different from trypsin but are present in normal trypsin preparations, may produce severe damage to the cell (119, 120).

CARRIER DETECTION

Developing in parallel with advances in prenatal diagnosis have been the methods to detect the carrier state for an increasing number of genetic disorders. In a larger sense, prenatal diagnosis really begins with heterozygote detection, especially as it involves the inborn errors of metabolism. There has been no choice in the past but to await the tragedy of an index case, and then monitor subsequent pregnancies for that specific biochemical disorder. Someday routine detection of carriers will anticipate this event and indicate prenatal monitoring for all the pregnancies of an "at risk" couple. For most metabolic disorders further technologic developments are necessary before heterozygote detection is appropriate on a mass scale. Furthermore, it is desirable that the pattern of inheritance is established, the disease confined to a recognized population group, carrier detection on a large scale being reliable, rapid, and simple, and prenatal diagnosis of the homozygous condition possible. Tay-Sachs disease has been the first disorder to fit these criteria, and Dr. Michael Kaback's pioneering screening program in Baltimore attests to the practicality of this approach.

In Table 1, many other disorders are shown which are candidates for postnatal heterozygote detection as well as prenatal diagnosis. Other more common problems such as sickle cell trait, the hemoglobinopathies, and the hyperlipoproteinemias are already suitable for mass screening. Such mass screening programs will inevitably introduce many logistic, technical, legal, moral, and other problems. Still, in the final analysis, all will probably agree that heterozygote detection is indeed the ultimate essence of antenatal genetic diagnosis.

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TREATMENT OF ACUTE LYMPHOBLASTIC LEUKEMIA[1]

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The treatment of acute lymphoblastic leukemia in the last decade can be said to have reached a revolutionary phase. Prior to that time, the disease was considered to be uniformly fatal and treatment was being given essentially for palliation. These concepts have changed and now the well-informed investigator is employing acute leukemia protocols which are aimed at cure rather than palliation. This revolution in both thought and method of treatment has been achieved to a large extent by the following:

1. The realization that an occasional case of acute leukemia could be apparently cured by single drug or simple combination therapy.
2. The demonstration that, by using readily available kinetic data in some transplanted mouse leukemias, specific dosage schedules could be devised to cure the disease in mice with a single drug.
3. The demonstration that such kinetic data could be procured in human acute leukemia to suggest better drug schedules.
4. The demonstration that combinations of drugs could be more effective than a single agent in both mouse and human leukemia.
5. The concept of total care of the patient including supportive measures, such as platelet and leukocyte transfusions, newer and better antibiotics, and protective environment which allows more intensive therapy.
6. The demonstration that Burkitt's tumor, a very rapidly growing solid tumor in African children that is somewhat analogous to acute leukemia, is curable in 50 percent of all patients and better than 76 percent of those whose disease is diagnosed early and is still small in total tumor volume (1, 2).

In this review, we will not attempt to cite all the articles on the treatment of acute leukemia but only those that have contributed to this revolution in thinking and results.

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LONG-TERM SURVIVORS

Data suggesting that leukemia is a curable disease was presented in 1964 (3) when it was shown that some 71 patients with acute leukemia collected from hematologists throughout the world had survived 5 years or more from the beginning of their disease and some of these had survived as long as 14 years. By 1967, this series had grown to 153 (4), and these same patients by 1971 showed 77 of 127 children and 16 of 31 adults alive and well with no evidence of disease 9 to 21 years from the diagnosis of their disease. Life-table analysis of these figures show that if a patient with acute leukemia survives 5 years, he has at least a 50 percent chance of surviving indefinitely (5). Some of the more recent figures which will be cited later suggest that if a patient is treated intensively, and remains in his initial complete remission for 3 years, he is likely to remain so indefinitely.

It should be noted that this series of 158 patients provides only the numerator of a fraction with an unknown denominator, i.e., the substrate of patients with acute leukemia from which these 158 5-year survivors were derived. Thus, it is impossible for this series to provide any indication of the incidence of 5-year survivals. The object of this study, however, was not to obtain such incidence figures but rather to indicate that long-term survivors did occur in acute leukemia and to pinpoint who and where these patients were in case immunologists or other investigators wished to do special studies on these remarkable cases. The great majority of these cases were acute lymphoblastic leukemia, but acute myeloid and acute monocytic leukemia were also represented. The majority were normal or low-count leukemias but occasional high-count leukemias were also included. For those 93 patients showing no evidence of leukemia at present the only therapy, with possibly one or two exceptions, consisted of 6-mercaptopurine, methotrexate, or steroids, or combinations thereof. In most of the disease-free patients, treatment has been discontinued for periods varying from 1 to 20 years.

ACCESSION OF THERAPEUTIC AGENTS

The accession of new compounds for the treatment of acute lymphoblastic leukemia started slowly, but the past decade and particularly the last five years have seen an ever increasing crescendo in this accession. Before 1947 there were no drugs that were of any significant use in the treatment of acute lymphoblastic leukemia. During 1947 and 1948, however, Farber et al (6) demonstrated that remissions could be achieved in children with acute leukemia by the use of the folic acid antagonist 4-aminopteroylglutamic acid (aminopterin) (7), and following these discoveries several other compounds acting as folic acid antagonists were also proven to be effective in this regard. It is generally agreed, however, that 4-amino-N^{10}-methylpteroylglutamic acid (methotrexate) (8) is the most satisfactory of the derivatives to date and that none of the other 4-amino-PGA or 2,4-diamino-5-phenylpyrimidine derivatives has any advantages over methotrexate.

The next group of agents to be discovered in the treatment of human acute leukemia were the adrenal steroids and ACTH. These findings were based on the pioneer work of Heilman & Kendall in 1944 (9) who showed that a lymphosarcoma in mice could be inhibited in growth, or the developed tumor caused to regress, by subcutaneous injection or feeding of compound E (cortisone) by mouth. Pearson, Eliel & Rawson (10) demonstrated the beneficial effects of ACTH in patients with chronic lymphocytic leukemia and then in acute lymphoblastic leukemia and, independently, Farber et al (11) showed the same effects in acute lymphoblastic leukemia. These investigators also demonstrated the similar beneficial effects of cortisone.

In 1951 the studies of Elion, Hitchings & VanderWerff (12) had demonstrated the anti-purine effects of 6-mercaptopurine (6-MP), and clinical studies at Memorial Sloan-Kettering Center (13) showed it to be beneficial in acute lymphoblastic, chronic myeloid and, to a lesser degree, in acute myeloid leukemia. 6-MP was active in cases whose disease had become resistant to methotrexate or steroids, as well as in previously untreated patients. Several other purine derives were tested clinically over the next few years, such as thioguanine (14), 6-chloropurine (14), and the imidazole derivatives. None seemed to be more effective than 6-MP in acute lymphoblastic leukemia, although recent studies seem to indicate that thioguanine in combination with 1-β-D-arabinofuranosyl cytosine (ara-C) is more effective than 6-MP both in the mouse and in the patient (15–18). One other advantage of thioguanine over 6-MP is that in some patients with acute lymphoblastic leukemia who have bulky disease with or without a pretreatment elevation of uric acid, allopurinol must be given to prevent hyperuricemia (19). Allopurinol does not affect the metabolism of thioguanine, whereas it does have a marked effect on 6-MP metabolism which makes it mandatory to give a much smaller dose of the latter.

The next agent to have beneficial effects in acute lymphoblastic leukemia was cyclophosphamide (Cytoxan) (20). Although this is an alkylating agent, it appears to be effective in producing occasional remissions in acute lymphoblastic leukemia, whereas the other alkylating agents such as nitrogen mustard or chlorambucil, have not been useful in this regard. As was the case with 6-MP and methotrexate, cyclophosphamide is more useful in the maintenance of remission rather than in the induction phase (21).

A new compound of great value in the induction of remission, however, is vincristine which was demonstrated to be active in acute lymphoblastic leukemia in patients in 1963 (22–24). Its clinical trial stemmed from the studies of Johnson et al (25) who demonstrated that a crude extract of *Vinca rosea* would produce prolongation of survival time and some cures in mice with P1534 leukemia. A chemical fractionation demonstrated that in this extract there were two discrete, highly active compounds (26): vinblastine which has proved to be of great value in the treatment of Hodgkin's disease and lymphosarcoma (27), and vincristine which is useful in both diseases (23, 28) but also in acute lymphoblastic leukemia (22–24).

The next agent to become available was 1-β-D-arabinofuranosyl cytosine (ara-C, cytarabine) (29). This drug, which is an antagonist of deoxycytidine and differs from this compound only in that it has arabinose in the sugar moiety instead of deoxyribose, presumably acts by inhibiting DNA polymerase and also, to a lesser degree, by inhibiting the reduction of cytidine diphosphate to deoxycytidine diphosphate. It was first shown to have antileukemic activity by Evans et al (30), and its clinical activity in lymphosarcoma was demonstrated by Talley & Vaitkevicius (31) and by others in leukemia (32, 33). The studies by Skipper et al (34) showed it to be extremely schedule-dependent, and after these data were available better clinical results were obtained. Ara-C is active in acute lymphoblastic leukemia (32, 33, 35, 36), but is even more important in acute myeloid leukemia (32, 33, 35, 37, 38) where it has become the mainstay of treatment, particularly in combination with the purine antagonist, thioguanine (16, 18), or with Cyclophosphamide (39).

A new departure in therapeutic drugs was the simultaneous development of daunomycin (40) and of rubidomycin (41), antibiotics which were shown to have antitumor and antileukemic effects in animals and to be useful clinically by Tan et al (42), Bonadonna et al (43), and Bernard et al (44), respectively. Later biochemical studies have shown that these two compounds are identical and they are now given the generic name, daunorubicin. The drug is useful in inducing remissions in acute leukemia and is also valuable in combination therapy. More recently, a derivative of daunorubicin, adriamycin (45), has shown even greater effects in mouse leukemia with less toxicity than daunorubicin (46), and has been shown to be active in patients with acute leukemia by both Bonadonna & Monfardini (47) and Tan et al (48). Continued use of daunorubicin has been associated with myocardial damage (49), whereas this has been noted to a much lesser extent with adriamycin (47, 48).

The development of L-asparaginase as an antileukemic agent stemmed from the original discovery of Kidd in 1953 (50) that there was a factor in guinea pig serum which inhibited the growth of certain mouse lymphomas. Further careful studies by Broome (51) culminated in 1961 in the demonstration that this antileukemic factor was indeed L-asparaginase. Following this, Mashburn & Wriston (52) extracted an asparaginase from *E. coli* with antileukemic activity which opened the way for therapeutic supplies of the drug. The original studies by DeBarros et al (53) in melanoma, and Dolowy et al (54) in acute leukemia suggested some slight clinical effects of low doses of L-asparaginase derived from guinea pig serum. The work of Hill et al (55) and Oettgen et al (56) demonstrated conclusively that remissions could be achieved with large doses of asparaginase derived from *E. coli*. Now, after many hundreds of patients have been treated (57–60), it appears that asparaginase will produce 50 to 70 percent remissions in acute lymphoblastic leukemia, but these remissions are of relatively short duration averaging three months, and resistance develops rather rapidly. For this reason and the fact that combination therapy with asparaginase is very successful in mice (61–63), it is probable that asparaginase is best used clinically in combination with other drugs.

New drugs which are strikingly effective against mouse leukemia and are presently undergoing clinical trial are 5-hydroxypicolinaldehyde thiosemicarbazone (5-HP) (64–67), 5-azacytidine (68, 69), and *cis*-platinum diamminodichloride (70, 71).

MENINGEAL LEUKEMIA

With the increasing life span of patients with acute lymphoblastic leukemia, there has been an increased incidence of meningeal involvement. This is particularly true since most of the agents which are used for the clinical treatment of this disease do not penetrate the cerebrospinal fluid, and therefore any wandering leukemic cells find sanctuary there from the therapeutic effects of the drug injected systemically or taken by mouth. It was demonstrated in 1958 by Whiteside et al (72) that methotrexate could be injected intrathecally without serious side effects and that extremely high levels of methotrexate could be maintained in the spinal fluid. This proved to be excellent treatment for the disease as long as it was limited to the meninges. Deeper infiltrations responded less well, and for these total cranial X ray was generally considered preferable. Ara-C has also been shown to be well tolerated and of value when given intrathecally either alone or alternating with methotrexate (73). One of the problems with repeated intrathecal injections is the formation of a false sac so that adequate quantities of methotrexate or ara-C do not get into the total spinal fluid. A treatment technique has been devised by Ommaya (74) in which a small plastic reservoir is located under the skin of the scalp with a plastic catheter leading through a burr hole in the skull to the lateral ventricle. In this way it is possible with minimal trauma to the patient to give frequent injections of either drug directly into the ventricular fluid. All of these forms of treatment have been useful in the treatment of the developed disease, but more recently both intrathecal methotrexate and total cranial or cranio-spinal axis radiation have been suggested as prophylactic measures. The results reported by Pinkel (75), Holland (76), and Tan et al (77) strongly suggest the great value of some form of prophylactic therapy against meningeal leukemia. The studies of Aur et al (78) have demonstrated this particularly well in a series of patients in which 2400 r of cranio-spinal radiation was randomized against no radiotherapy. There were two CNS relapses in the 45 irradiated patients as against 27 CNS relapses in the 49 nonirradiated patients. Such measures are now being incorporated into most of the large group protocols for the treatment of this disease. It is generally agreed that prophylactic therapy is much more successful in preventing meningeal involvement than the same treatment is in curing the disease once it has developed to clinical proportions (78).

KINETICS AND PHARMACOLOGY

In addition to the rapidly accelerating accession of new drugs, there has been considerable pharmacologic study on existing drugs which has suggested how these compounds might be used in a more effective manner. Initial studies with methotrexate by Goldin et al (79) showed that the drug was more effective when given every second or every third day than when given daily, and the first curative use of methotrexate in choriocarcinoma by Li, Hertz & Spenser (80) employed methotrexate in short four- to five-day courses of massive daily doses and then provided an interval of two to four weeks for the recovery of the normal tissues. Similarly, the initial studies on methotrexate in Burkitt's tumor (81) showed the definite advantage of this same intensive dosage for four days at 2- to 4-week intervals, as contrasted to daily doses of methotrexate. One of the most successful protocols of Acute Leukemia Group B (82) employs intensive five-day courses of methotrexate every two weeks. Other clinical studies initiated by Selawry (83) in 1965 demonstrated that methotrexate maintenance therapy given at 30 mg/M^2 twice weekly produced much longer remissions than when the drug was given on a daily basis, and even produced an occasional five-year survivor free of disease. Cyclophosphamide had been shown in mouse leukemias to be more effective when given in a weekly dose than when given daily (84). Similarly, in Burkitt's tumor a massive dose of 40 mg/kg given once every three to four weeks for a total of six doses was much more effective than the small daily doses (85). In combination therapy, Simone & Pinkel (21) have reported excellent long-term results with cyclophosphamide given at 200 mg/M^2 weekly in combination with methotrexate and 6-MP.

Perhaps the most schedule-dependent of all the chemotherapeutic agents is ara-C which did not cure L1210 leukemia at any dose given on a daily basis, but which produced a high percentage of cures in early leukemia when given every 3 hr for eight doses, with this course repeated every fourth day (34, 86).

The rationale behind the greater success of ara-C in mouse leukemia when given every 3 hr over a 24-hr period, as shown by Skipper, Schabel et al (34, 87, 88), is that in this way adequate concentrations are available during the whole period of two cell cycles, so that almost all (more than 99 percent) of the cells during that period presumably will go through the DNA synthesis phase and so be susceptible to damage by ara-C. To accomplish this in the cells of human leukemia, where the cell cycle is longer, 24 to 96 hr (18, 89–91), and where a much smaller percentage of cells are in the proliferative phase, probably requires a coverage with adequate dosage concentrations over a period of at least five to nine days and a repetition approximately every three weeks for a protracted period of time in order to destroy all cells in the proliferative phase (91). Compounds such as cyclophosphamide are not limited to the DNA synthesis phase for their activity and therefore are effective on the nonproliferating cells as well as against those in the active state of proliferation (92, 93). Thus, the use of short, intensive courses of antimetabolites with intervals for the recovery of normal cells, interspersed with single doses or short courses of less cycle-dependent drugs such as cyclophosphamide, vincristine, and prednisone, should help eliminate not only leukemic cells in the proliferative state but also those resting cells in the G_0 or prolonged G_1 phase. The exact extrapolation of any mouse dosage schedule to man is difficult (94), but administration of a large dose of ara-C continuously for five days (95, 96), or by intravenous push every 6 hr or every 8 hr for three to five days alone, or with cyclophosphamide (39, 96), or the every-12-hr administration of ara-C and thioguanine for five to nine days (18, 97) with a two- to three-week rest interval have all demonstrated advantages over the once-daily administration of this drug or combination.

Studies of the clinical pharmacology of ara-C, reported by Ho and Frei (98, 99), have shown that the plasma disappearance curve of ara-C in man is biphasic, with an initial fast phase (mean half-time 12 min) and a slower second phase (mean half-time 111 min). Seventy percent of the dose is excreted within 24 hr, 8 percent as ara-C, and the remainder as the inactive metabolite, ara-U. The deamination of ara-C to inactive ara-U occurs mainly in the liver, but also in the leukemic cells per se. The usual mechanism of resistance to ara-C in mouse leukemia is the loss of the nucleoside kinase in the resistant cells (100, 101), but recent studies by Steuart & Burke (102) have demonstrated an increased content of deoxycytidine deaminase, the enzyme which converts ara-C to ara-U, in cells of patients whose leukemia had become resistant to ara-C, as contrasted to those same cells at a time when the patient's disease was sensitive to the drug.

INCIDENCE OF FIVE-YEAR SURVIVAL

As was previously mentioned in the series of cases of long-term survivors collected by our group, no figures were available for the incidence of five-year survivors (4). The first large-scale series with data on such incidence was that of Zuelzer (103) who treated 229 successive cases of acute lymphoblastic leukemia in children with prednisone and 6-MP for induction of remission, and then alternated indefinitely between three months of daily oral methotrexate and three months of daily 6-MP for maintenance therapy. In this large series he was able to report 4.3 percent 5-year survivors. A greater improvement in long-term survival, however, began with the use of intensive therapy and particularly the use of intensive combination therapy based on the many studies of combination therapy and total leukemic cell kill of Skipper, Schabel et al (24, 87, 88), Venditti & Goldin (104), and others (61, 62). Selawry (personal communication), in a small series treated with methotrexate 30 mg/M^2 given twice weekly intravenously, had three of 21, or 14 percent, surviving 5 years. Frei, Freireich, and Henderson et al, using the VAMP (105) and BIKE (106) program, had about the same results. The POMP (107) program consisted of intensive courses of four different drugs, repeated approximately once monthly for 14 months. All of these combinations were based on the theory that massive doses of several different chemotherapeutic agents with differing and therefore not additive toxicities, given over 5 to 10 days with an adequate rest interval to allow recovery of normal cells, would be more effective than daily doses of a single drug or even of combinations. Studies of this type have been improved upon by Leukemia Group B. The results shown by Holland (82) in 1969 demonstrated the progress that they had made, ranging from almost no five-year survivors before 1960 to approximately 2 percent in 1960–61, 4 percent in 1962–63, and 8 percent by 1964–65. In addition to this, the 230 patients treated in 1966–67 showed a much higher percentage of survivors at 36 months than in the previous series. One small selected group of these had over 70 percent survivors at 24 months. A more recent updating of this small selected series by Holland (76) has shown that those patients receiving induction with vincristine and prednisone, followed by consolidation with an eight-month course consisting of five daily doses of methotrexate every two weeks, including intrathecal methotrexate approximately once a month, followed by twice weekly methotrexate as maintenance therapy, gave approximately 60 percent survivors at 55 months. A more recent study, 6801, suggests that the addition of asparaginase to the induction regimen and of 6-MP to the maintenance protocol may be improving results (76, 122).

Pinkel (75) and the group at St. Judes Children's Research Hospital have reported recently on a group of 41 children in studies I to III treated initially with prednisone and vincristine for remission induction, followed by oral administration of 6-MP daily, and vincristine and cyclophosphamide and/or methotrexate weekly. Low doses of radiation therapy, 500 to 1200 r, were given prophylactically to the cranio-spinal axis. In addition, in order to avoid long-term toxicity, some children were randomized to half-dose chemotherapy. These had a significantly shorter survival and time in complete remission than those randomized to full-dose chemotherapy. Seven of these children have remained in complete remission for more than five years and have not received any therapy for one and one-half to three and one-half years. Pinkel states (75) that approximately two thirds of the children entered into later studies in 1967 and 1968 are presently in continuous complete remission and have been for two years or more. He also maintains that, since the majority of children in the original studies I to III who remained in complete remission longer than two years were free of disease at five years, it is possible that the five-year cure rate of childhood acute lymphoblastic leukemia in this most recent series may reach the 50 percent level. In a later protocol, using vincristine and prednisone for induction, 6-MP 60 mg/M^2 daily, methotrexate 30 mg/M^2 weekly, and cyclophosphamide 200 mg/M^2 weekly by mouth for consolidation and maintenance, Aur et al (78) and Simone (21) tested the relative value of 2400 r prophylactic cranio-spinal irradiation as compared to no radiation until CNS leukemia was demonstrated. At the time of writing, 34 of 45 patients receiving radiation were in complete remission as against 16 of 49 who had not been irradiated.

REINDUCTION OF LEUKEMIA

In addition to the problem of total cell kill which we have been discussing, there is another problem which has recently given some indication of importance, i.e., the possibility that in some cases, although one may achieve complete leukemic cell cure, there may remain a virus or other agent which is potentially capable of reinducing the disease. Thus, a patient who again becomes leukemic after five years of complete remission may be relapsing not by regrowth of a few resistant cells left over from the original treatments, but rather from new leukemic cells induced by the same agent which caused the original disease. There is precedence for this view in breast cancer where a patient with carcinoma in one breast, removed by radical mastectomy, may be entirely cured only to have a second primary develop in the other breast a few years later. The studies by Glynn et al (108) in transplanted Moloney leukemia demonstrated the possibility of total cell cure by cyclophosphamide only to have the disease recur six months later by reinduction from the Moloney virus contained in the original cell inoculum. Similarly, Skipper & Schabel (87, 88) were able to cure spontaneous AKR leukemia, which is Gross virus-induced, with a significant number of 50-day survivors, only to have them suffer another reinduced spontaneous leukemia at a later date. Perhaps the most cogent argument in favor of reinduction of human leukemia by a virus or other agent was supplied by serendipidy. Thomas et al (109) irradiated a leukemic female child with 1000 r total body radiation to destroy her leukemic cells and then transplanted marrow from an HLA-matched brother. The brother's cells grew well and produced a normal blood and marrow picture for approximately two months. At the end of three months, however, the patient was in complete relapse and the leukemic cells were shown by chromosome analysis to be entirely from the cells of the normal brother. This would suggest that whatever agent produced the leukemia in the girl originally was still present and capable of inducing leukemia in the transplanted cells from her healthy brother (109).

In the series of 158 long-term survivors which we have collected, there are three patients who have developed leukemia after 7 to 16 years of complete remission and after 4 to 8 years off therapy (110). Similarly, Clifford et al (111) have reported two patients with Burkitt's tumor in whom a second tumor appeared after discontinuing therapy for four disease-free years. On the basis of what is known of cell kinetics at the present time, it is difficult to explain how these manifestations of disease could have taken so long to arise from even a single viable leukemic or Burkitt's tumor cell remaining at the end of treatment. This leads to the suggestion that, in addition to therapy aimed at killing the leukemic cells, it may be necessary in the future to employ also therapy aimed at a potential virus. A possible ray of hope along this line lies in the inhibitors of RNA-directed DNA polymerase (reverse transcriptase) which have been reported recently.

INHIBITORS OF REVERSE TRANSCRIPTASE

Most of the naturally occurring, virus-caused leukemias in animals are caused by RNA viruses, and for many years attempts have been made to understand how an RNA virus could so affect the genetic information of a cell that the neoplastic change, once initiated, would be transmitted from cell to cell. The studies of Temin (112), Baltimore (113), Green (114), Spiegelman (115), and Gallo et al (116) have demonstrated that all of some 30 oncogenic viruses so far tested—but only two viruses which have not so far been shown to be oncogenic—contain a bizarre enzyme known as RNA-dependent DNA polymerase (RDDP) or reverse transcriptase. Because of this enzyme the information contained in the RNA virus is transmitted to the DNA of the infected cell instead of the usual procedure in which DNA directs the formation of RNA which, in turn, directs the formation of proteins. When Gallo (116) reported that a similar RDDP could be demonstrated in cells of human acute lymphoblastic leukemia but not in phytohemagglutinin-stimulated normal lymphocytes, this was interpreted as a possible indication that the cells contained an oncogenic virus. Because of the need for careful purification of the enzyme and of the varying specificities of the natural and artificial templates employed, the picture is complicated and it is not possible to say for certain at the present time whether the presence of the enzyme in human leukemic cells reported by Gallo (116) is a true indication of an oncogenic virus (117).

Studies by Gurgo et al (118) have shown that the RDDP from avian and rodent leukemia viruses is inhibited by various derivatives of rifampicin, in which the N-methyl of the aminopiperazine moiety is removed or substituted by a benzyl, but is not inhibited by the parent compound. Gallo, using his enzyme from human acute lymphoblastic leukemia cells (116), has reported similar results for the N-demethyl rifampicin at levels at which the parent rifampicin was ineffective. Various streptovaricin derivatives have also been shown by Brockman et al (119) to inhibit the RDDP from avian and murine oncogenic viruses. More recently, Carter et al (120) and Borden et al (121) have shown the ability of the streptovaricin derivatives to inhibit focus formation by the Moloney leukemia-sarcoma virus and, when fed in the diet at 1500 mg/kg/day, to inhibit partially the splenomegaly induced by the Rauscher leukemia virus. These findings suggest, as a future possibility, the use of some such antiviral agent after total leukemic cell kill has been achieved by conventional chemotherapy. That these derivatives of rifampicin and streptovaricin might also be active against leukemic cells is suggested by our studies (110) of these compounds for their ability to inhibit growth in vitro and to sterilize proliferating and nonproliferating L5178Y and P815 mouse leukemic cells in cell culture after 48-hr incubation with the drug in vitro, as demonstrated by bioassay into isogeneic recipient mice.

The 4-benzyl and the 2′,6′-dimethyl-4-benzyl rifampicin derivatives were both more active in sterilizing these cells than the parent rifampicin or the N-demethyl derivative. Of the streptovaricin derivatives studied, the crude streptovaricin extract (U 7750) was the most active. It is of considerable interest that with such compounds the temperature of incubation, and consequently the proliferative state of the control cells as shown by ^{3}H-TdR uptake, made only a slight difference, so that sterilization was achieved by only a three-fold higher concentration at 22°C than at 37°C (110). The levels that would sterilize the leukemic cells at 37°C are roughly comparable to those reported to be effective in inhibiting the activity of the enzyme by Gurgo et al (118) and by Carter et al (120). Whether these rifampicin derivatives will have any effect on mouse leukemia in vivo or in patients awaits the production of larger quantities of these very scarce derivatives.

The cumulative experience of the various cooperative groups (21, 75–78, 122) studying acute leukemia suggests that optimum therapy for acute lymphoblastic leukemia might call for the induction of a marrow remission with vincristine and prednisone, with possibly a short course of asparaginase added, followed by consolidation with methotrexate 15 mg/M^2 daily for five doses, repeated every two weeks for five courses. The fifth dose of methotrexate would be given intrathecally instead of by mouth on the first, third, and fifth courses. Following this would come a two-week period of reinforcement treatment using vincristine 2 mg/M^2 weekly×2, prednisone 40 mg/M^2 daily by mouth, and daunorubicin 60 mg/M^2 in a single dose. At the end of this time, intensive therapy aimed at destruction of any leukemic cells remaining in the CNS might be given as either 2400 r of cranio-spinal radiation over a three-week period, 2400 r of cranial radiation plus intrathecal methotrexate, or only intrathecal methotrexate 15 mg/M^2 twice weekly for three weeks. Following this, the patient would be put on maintenance therapy consisting either of 6-MP 60 mg/M^2 daily, methotrexate 30 mg/M^2 weekly, and cyclophosphamide 200 mg/M^2 weekly by mouth (21, 78), or 6-MP 90 mg/M^2 daily and methotrexate 15 mg/M^2 weekly by mouth (76, 122) for 6 to 10 weeks followed by two weeks of reinforcement therapy as noted above, after which the maintenance would be repeated for 6 to 10 weeks and reinforcement therapy for another two weeks, for a total of at least eight months. At that time, the child should either continue on the original maintenance regimen or switch to methotrexate 30 mg/M^2 twice weekly by mouth. Tan et al (123) have reported on another regimen using vincristine, prednisone, and adriamycin for induction, prophylactic intrathecal methotrexate, then ara-C, thioguanine, L-asparaginase, and 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) for consolidation, and hydroxyurea, methotrexate, and cyclophosphamide plus some of the previous drugs for maintenance. Although the short-term results appear to be excellent, no long-term comparison is yet available with the above types of treatment. After two and one-half to three years of complete remission under this treatment it would appear reasonable to discontinue therapy. Data supporting this conclusion derive from the report of Pinkel (75) that those patients remaining in continuous complete remission on therapy for two and one-half to three years have not relapsed in the ensuing two to three years off therapy, and from Krivit et al (124) that in 14 patients randomized to maintenance or no maintenance therapy after three years of continuous complete remission, discontinuing therapy did not influence the relapse rate. The role of BCG at this time has been discussed by Mathé (125). Whether BCG scarification should then be used in an attempt to increase host defenses against any leukemic cells remaining after this therapy is still undecided. Certainly, if BCG is to be used, this would be the time that it would have the most chance of effectiveness.

These various protocols make use of all the agents active in acute leukemia including BCNU, hydroxyurea, and the combination of ara-C and thioguanine. Since ara-C, however, at the present time appears best given twice or thrice daily intravenously, it is less adaptable to maintenance protocols than are the other agents which may be given by mouth. A slow-release form of ara-C in the form of the palmitate, known as palmo ara-C, has shown improved results in mice (126) and is now undergoing clinical pharmacological studies (Frei, E., III, personal communication). This should greatly simplify the administration of ara-C and make this exceptionally valuable compound available for outpatient use without sacrificing maintained adequate blood levels. Similarly, tetrahydrouridine, an inhibitor of deoxycytidine deaminase, prevents the deamination of ara-C to ara-U and so allows the maintenance of adequate levels of ara-C for a significantly longer time. The delayed toxicity of BCNU makes it somewhat difficult to adapt to sequential protocols, although both this agent and hydroxyurea are capable of crossing the blood-brain barrier. 5-HP is active against mouse leukemia (64, 65), particularly in combination with adriamycin, and has produced short, partial remissions in children with acute lymphoblastic leukemia (66), but the iron-chelating effects of this drug and the severe nausea and vomiting produced make it difficult to adapt to maintenance therapy. Another agent, 5-azacytidine (67 68), a metabolic antagonist of cytidine (69), has shown marked activity against mouse leukemias including those resistant to ara-C.[2] Similarly, *cis*-platinum diamminodichloride is very active against L1210 (70, 71), P815[2], and L5178Y[2] mouse leukemias including lines resistant to ara-C and vincristine.[2] All these compounds are undergoing clinical investigation in acute lymphoblastic leukemia, but their exact role alone or in combination therapy aimed at cure is still unclear.

The treatment of acute lymphoblastic leukemia should be a team effort (82) in which the diagnosis is made by the patient's pediatrician or internist, the initial intensive treatment and planning of the whole course of therapy done in a center specializing in this type of treatment of acute leukemia, and the maintenance therapy carried out by the patient's pediatrician or internist in close cooperation with the center. Only with such close cooperation between the patient, the referring physician, the center, and the patient's family can optimum results be achieved.

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[2] Burchenal, J. H. and Dowling, M.D., Jr., unpublished observations.

The hope in setting up such an intensive combination protocol is eventually to reduce the leukemic cell population from its original approximately one trillion (10^{12}) cells down to zero, and to do this with the least interference to the patient's normal life during the procedure. These two are not always compatible, and it must be borne in mind that if there has to be a compromise between the optimum leukemic cell kill and the convenience of the patient, the cell kill must come first. This is a gamble for high stakes: the continued and indefinite survival of the patient. Any diminution in treatment, or any failure to pursue the treatment relentlessly, may compromise the patient's chances of survival. It is very easy with many of the relatively nontoxic agents available, such as vincristine, prednisone, and 6-MP, to produce a remission in which the child or adult goes back to complete health, and in which perhaps 99 percent or even 99.9 percent of the leukemic cells are destroyed. One must always keep in mind, however, that with 99 percent of the leukemic cells destroyed and none recognizable in either the peripheral blood or the marrow, there still remains a 10 billion leukemic cell burden in the patient, any one cell of which surviving can alone give rise to a fatal outcome of the disease. Thus, the most strenuous measures are necessary and must be undertaken if one is to achieve what now appears in many patients to be an attainable goal—the cure of the patient with acute lymphoblastic leukemia.

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RED CELL ENZYME DEFICIENCIES AS A CAUSE OF HEMOLYTIC DISORDERS 7031

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INTRODUCTION

The human red cell, biologically speaking, is intriguingly but deceptively simplistic. Freely floating, separable from contaminating cell types, devoid of a nucleus and cellular organelles, restricted in its energy needs and in the mechanisms for meeting them, it can be biopsied repeatedly with safety and offers many lures to the investigator. In recent years its enzymatic machinery has been explored with increasing intensity, and a variety of genetically determined inborn errors have been demonstrated (Valentine, 1, 2; Jaffé, 3). Some have been associated etiologically with hereditary hemolytic disease, and some have clarified mechanisms of what formerly were regarded as idiosyncratic reactions to drugs. Some have provided a convenient diagnostic tool for defining diseases not characterized by hematopoietic abnormalities. In still others, the definition of genetically determined enzymopathy has been a curiosity with no counterpart in clinical disease.

RED CELL METABOLISM

The human erythrocyte normally derives most of its energy needs from the conversion of glucose to lactate via the Embden-Meyerhof pathway. In simplest terms, a mole of glucose thus metabolized could result in a net gain of 2 moles of high-energy ATP. Two moles of the pyridine nucleotide NAD (DPN) are reduced to NADH (DPNH) and reoxidized. However, the ATP-generating reaction mediated by phosphoglycerokinase (PGK) can be bypassed if triose is converted to 2,3-diphosphoglycerate (2,3-DPG) and then returned to the mainstream of glycolysis as 3-phosphoglycerate (3-PG). This alternative pathway (Rapoport & Luebering, 4) is important in human and certain mammalian erythrocytes. Therefore, anaerobic lactate production results in an average net gain of between 0 and 2 moles of ATP per mole of glucose metabolized, depending on the amount of triose shunted through the 2,3-DPG pool.

Glucose-6-phosphate (G-6-P), the product of the initial phosphoglyation of glucose by hexokinase (HK), may be alternatively metabolized through the oxidative hexomonophosphate (HMP) shunt. G-6-P, through the action of glucose-6-phosphate and 6-phosphogluconate dehydrogenase (G-6-PD and 6-PGD), becomes pentose. CO_2 is evolved, and NADP (TPN) is reduced to NADPH (TPNH). Pentose is interconvertible with intermediates of the anaerobic, Embden-Meyerhof pathway. The proportion of glucose traversing the HMP shunt is dependent upon (*a*) the activity of shunt enzymes and (*b*) the reoxidation of NADPH to NADP. The latter is essentially dependent on the NADPH-linked reduction of oxidized glutathione (GSSG) by glutathione reductase (GSSG-R). The availability of GSSG, in turn, depends on the rate of oxidation of intracellular reduced glutathione (GSH). This varies widely with environmental factors including oxidative stresses induced by certain drugs. The percentage of HMP-metabolized glucose is, therefore, itself widely variable.

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The human erythrocyte also possesses a wide variety of other enzymatic machinery. It has a limited but still presumably important capacity to renew its adenine nucleotide, pyridine nucleotide, and flavin nucleotide cofactors. It possesses a true acetylcholinesterase, certain enzymes of pyrimidine, galactose, glycogen, and urea metabolism, as well as methemoglobin reductase, catalase, glyoxalase, and many other activities. Many sequences are rudimentary, and others are of importance chiefly as mirrors of activity in other tissues where their presence has more relevance. The red cell requires comparatively little metabolic fuel. It must, however, pump cations against gradients, resist methemoglobin formation, maintain its shape and distortability and protect against oxidative denaturation of hemoglobin and crucial proteins.

CLASSIFICATION OF ENZYME DEFICIENCIES

Embden-Meyerhof pathway enzymes.—These now include the following recognized deficiency states (Valentine, 5): hexokinase (HK) (Valentine et al, 6): glucosephosphate isomerase (GPI) (Baughan et al, 7); phosphofructokinase (PFK) (Tarui and co-workers, 8); triosephosphate isomerase (TPI) (Schneider et al, 9); 2,3-diphosphoglyceromutase (2,3-DPGM) (Schröter, 10); phosphoglycerokinase (PGK) (Kraus and co-workers, 11; Valentine et al, 12); pyruvate kinase (PK) (Valentine and co-workers, 13; Tanaka et al, 14); and lactic dehydrogenase (LDH) (Miwa et al, 15). Severe deficiency of HK, GPI, PFK, TPI, 2,3-DPGM, PGK, and PK have been clearly associated with hereditary, nonspherocytic hemolytic anemia. PFK deficiency has been reported in two forms—one expressing itself solely as a hemolytic disorder, and the other as a very mild, compensated hemolytic process associated with a severe disease of muscle metabolism (Glycogenosis, Type VII). Partial deficiency of G-3-PD has likewise been reported as has deficiency of the enzyme catalyzing dephosphorylation of 2,3-DPG (Syllm-Rapoport et al, 16). The former (Oski & Whaun, 17), mentioned in a single report, requires confirmation as a cause of hemolytic disease. The latter was associated with a hemolytic process, but also a multiplicity of congenital anomalies, rendering interrelationships difficult to ascertain. Severe deficiency of LDH due to inability to synthesize one of the subunits of the enzyme has recently been documented (Miwa et al, 15). Interestingly, hemolytic anemia was not demonstrated, though certain secondary metabolic abnormalities were noted.

HMP shunt and related enzymes of GSH metabolism.—The first studied and most widely distributed red cell enzyme deficiency is that of G-6-PD (Carson et al, 18; Beutler, 19). Partial and severe deficiencies of 6-PGD are also known, but relationship to hemolytic disorders is less certain (Brewer, 20). GSH peroxidase (GSH-Px) deficiency (Necheles et al, 21) has been observed in two kindreds. In one, the proband had overt hemolytic anemia. The enzyme catalyzes the destruction of peroxides via the simultaneous oxidation of GSH. GSSG-R deficiency has been widely reported in subjects with extremely diverse clinical manifestations (Waller, 22). The majority of reports are obscured by recent information clearly showing that apparent GSSG-R deficiency may result from a deficiency of its cofactor, flavin adenine dinucleotide (FAD). The latter may reflect deficiencies in FAD synthesis and is, importantly, also very sensitive to nutritional status and riboflavin intake (Beutler, 19). GSH deficiency has also been documented in a few kindreds (Oort and co-workers, 23), some members of which have hemolytic anemia and almost total absence of red cell GSH or GSSG. In three kindreds (Boivin et al, 23a, and Mohler and co-workers, 23b) the enzyme catalyzing the second step in GSH synthesis, namely, GSH synthetase, was found to be deficient.

Other erythrocyte enzyme deficiencies.—Abnormalities in adenine nucleotide metabolism are only beginning to be appreciated. "High ATP" has been described in the absence of any clinical manifestations or any other thus far documented metabolic abnormalities (Brewer, 24), in the presence of genetically determined increases in PK but, again, without overt symptoms (Zürcher et al, 25) and in conjunction with 2,3-DPG phosphatase deficiency (16). We have recently observed hereditary hemolytic anemia in two subjects with high red cell ATP and substantial reductions in ribosephosphate pyrophosphokinase (RPK, or PRPP synthetase) (Valentine et al, 26). RPK catalyzes the production of 5-phosphoribosyl-1-pyrophosphate (PRPP) and adenosine monophosphate (AMP) from ATP and ribose-5-phosphate (R-5-P). PRPP is an important constituent of a number of reactions in purine, pyrimidine and nucleotide metabolism. Busch (27) has reported a clinically similar case, but it is not known if RPK was deficient or not. The syndrome of acatalasemia (Takahara, 28) is accompanied by virtually total absence of red cell catalase. Red cell enzyme deficiencies have also been demonstrated in such syndromes as galactosemia (Isselbacher et al, 29), oroticaciduria (Smith et al, 30), and arginosuccinic aciduria (Efron, 31).

Hereditary methemoglobinemia and red cell DPNH-methemoglobin reductase deficiency is a separate and fascinating story in itself (Jaffé, 32). The list is incomplete.

CLINICAL AND LABORATORY ASPECTS

General.—The diversity of manifestations of erythrocyte enzyme deficiencies and shortage of space limit discussion to the main features of those deficiencies characterized by hemolysis. Hemolytic anemia, when associated with Embden-Meyerhof pathway enzyme deficiencies, has been variable but often severe. Sharp exacerbations in anemia may accompany infection or stress. Reticulocytosis is present, hypotonic osmotic fragility of red cells in fresh blood is normal, the antiglobulin test is negative, no hemoglobinopathy is demonstrable, and usually splenomegaly is noted. The stained blood smear has only rarely shown unusual red cell shape changes except for the presence of variable numbers of irregularly contracted cells.

In G-6-PD deficiency, most subjects have hemolytic anemia only when challenged by certain drugs producing oxidative stress, by infection, or by fava bean ingestion. The deficiency affects more than 100 million people. Its distribution bears a relationship to that of falciparum malaria from whose ravages it may provide some protection. During episodes, hemolysis is intravascular, and characteristically only the older cells are destroyed. Chronic hemolytic anemia characterizes a small percentage of severely deficient subjects. 6-PGD deficiency is not nearly so well characterized and its clinical implications remain largely unelucidated. Near total absence of erythrocyte GSH and GSSG is characterized by moderate chronic hemolysis, and by exacerbations inducible by primaquine and other oxidative drugs. GSH-Px deficiency is rare, but may be accompanied by chronic hemolysis, Heinz bodies in the erythrocytes, and resembles G-6-PD deficiency in other respects.

Specific.—Enzyme deficiencies may be generalized or restricted, depending upon the isozyme pattern indigenous to various tissues. Thus, in the case of PFK, erythrocytes possess about one-half their activity in the form of "muscle isozyme" and about one-half in a form not possessed by muscle. In a severe disorder of muscle metabolism characterized by near total PFK deficiency, red cells have about 50 percent of normal PFK activity and there is only minimal, compensated hemolysis (Tarui et al, 8). In TPI deficiency the abnormality is widespread and the clinical manifestations include a severe, progressive neurologic disease, susceptibility to cardiac arrest or ventricular fibrillation, and perhaps diminished resistance to infection. In severe PGK deficiency, there are suggestions that neurologic dysfunction is present as well as hemolytic anemia. Tissue deficiency of some enzymes may not lead to disease if alternative metabolic pathways render it innocuous

It has already been mentioned that drug- and stress-induced hemolysis may occur with certain enzymopathies.

Treatment.—Aside from transfusion, the treatment of enzyme deficiency-induced hemolytic anemia is largely limited to splenectomy. The latter fails to correct the hemolytic process, but sufficient benefit may result to mitigate or eliminate transfusion requirements, improve significantly the level of circulating hemoglobin or, occasionally, be the difference between death and survival. Such partial benefit is best documented for PK deficiency, seems probable in GPI deficiency, and possibly may also be achieved in other deficient states in which convincing documentation is still not available. Interestingly, it appears that deficient reticulocytes in some circumstances may be more susceptible to destruction than older cells. Where drug challenge, stress, or fava bean ingestion precipitate hemolysis, treatment consists of avoidance of known offending agents.

METABOLIC ASPECTS

Enzymatic deficiences make for a diversity of metabolic epiphenomena only a few of which will be cited as examples of how nature may provide experimental conditions otherwise unachievable. For example, PK deficiency is associated with increases in a number of glycolytic intermediates proximal to the deficient step. Most striking is the elevation in red cell 2,3-DPG, a metabolite exerting significant effect on the hemoglobin oxygen dissociation curve. TPI deficiency is associated with striking increases in cell dehydroxyacetone phosphate (DHAP). GPI deficiency particularly impairs the reverse isomerization of fructose-6-phosphate (F-6-P) to G-6-P, and hence the ability to recycle F-6-P generated from pentose through the HMP shunt. LDH deficiency results in inefficient oxidation of NADH, and hence leads to aberrations in the NAD/NADH ratio. In other instances, ATP stability is imparied, overall glycolysis is less than expected, and membrane abnormalities may accompany disturbance in the energy-generating mechanisms.

GENETICS

Most of the erythrocyte enzyme deficiencies are autosomally coded, but G-6-PD and PGK are X-chromosome linked. In the latter two instances, hemizygous males and homozygous females are severely affected; heterozygous females, in accordance with the Lyon hypothesis (33), usually have intermediate manifestations, but may range from phenotypically normal to as severely affected as males. In autosome-linked deficiencies, the homozygote is usually phenotypically detectable. Heterozygotes in most instances have no overt clinical manifestations and are hematologically normal. Partial deficiency is definable by specific enzyme assay. While most phenotypically detectable deficiencies are rare, G-6-PD deficiency is common. Its high instance in the black and in certain non-Askenazy Jewish groups has been attributed to the beneficial effect of diminished parasitism of deficient cells by Plasmodium falciparum. Over 80 variants, characterized by differences in catalytic activity, stability, pH optima, kinetics, and electrophoretic mobility are already recognized. PK deficiency has likewise been clearly shown to involve a multiplicity of mutant alleles, presumably at the same locus, which are responsible for both qualitative and quantitative aberrations from normal (Tanaka & Paglia, 34). Other rarer deficiencies also exhibit polymorphism. Thus, more than one mutant allele has been demonstrated in GPI, HK and 6-PGD deficiency. Additional studies will certainly broaden evidence for the remarkable polymorphism of man.

THE REWARDS AND THE PROBLEMS

The definition of inborn errors in the human red cell has brought many rewards. In the case of G6PD and certain HMP shunt-related enzyme deficiencies, understanding of the problem and screening procedures permit avoidance of hemolysis-precipitating medications and events. Perhaps equally important, a large group of drug reactions has been removed from the nebulous realm of "idiosyncrasy." Their etiology is understood and separable from other noxious consequences of therapy or environmental exposures. The beneficial effect of splenectomy in certain deficient states, even though incomplete, is of obvious importance. Other studies have aided the population geneticist, and additional gene markers have been provided in the case of G-6-PD and PGK. The relationship of the G-6-PD deficiency to malaria has provided additional insight into how an otherwise disadvantageous gene may provide selective advantage in certain environments. In females heterozygous for G-6-PD deficiency, there has been opportunity to evaluate the uniclonal versus polyclonal origin of tumors from the enzyme pattern of metastatic foci. Certain other rewards, thus far only beginning to be exploited, include the opportunities afforded to study the subtle effects of abnormal distributions of a wide variety of metabolites on cell function. Highly integrated feedback and feed-forward mechanisms of control of cell function are difficult to study in contrived systems comparable to those resulting from a highly specific, inborn metabolic error. Further, such experiments of nature sometimes shatter time honored convictions which, while unsubstantiated, have achieved the status of near dogma. Who would have believed that nearly total absence of catalase or of red cell GSH would be so well tolerated by the human organism? Significant clinical effects accompany the latter, yet the order of catastrophe is far less than many would have anticipated.

The challenges of discovery have raised additional problems. What is the ultimate mode of destruction of the deficient cell? Why are there poorly explained dichotomies between clinical symptoms and enzyme activity as determined by in vitro assay? What is the role of intermediate accumulation in the total disease complex? What is the nature of membrane dysfunction which seems to accompany many deficient states, and which may perhaps be crucial in the terminal agonies of the cell nearing destruction? What is the role of the spleen in the various enzymopathies? What is the relative importance of deficiency in the localized membrane portion as compared to the soluble, intracellular component of a given enzyme? These and other questions provide a setting for future investigations.

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[2] Limitations in space and great diversity of material have made it necessary to limit greatly references cited. These have been largely confined to heavily annotated and current reviews, to original descriptions of deficiency states, or to selected key papers. Many highly important contributions are, therefore, unreferenced but can themselves be readily obtained from the bibliographies of papers cited.

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HUMAN PLATELET SENESCENCE

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Human platelets are anucleate cytoplasmic fragments derived from nucleated bone marrow megakaryocytes and circulate in the blood stream for 10 days. Platelets provide a dual function in hemostasis: (*a*) they repair tears or holes in blood vessels; (*b*) they provide a phospholipid surface (catalyst) for the interaction of certain coagulation factors, which serve to generate fibrin. It has been proposed that platelets are constantly utilized in the "continuous" repair of small breaks or tears in the blood vessels (1). This would imply random destruction of platelets, rather than ordered (i.e., one in which platelets disappear from the circulation after a finite duration of time.)

Platelet survival studies, performed with Cr^{51} or DFP^{32}, have provided two controversial types of "normal" curves depending upon the laboratory in which they were derived (Fig. 1A, B). Before explaining the controversy, a few comments on methodology are in order. Both isotopes are thought to label the entire population of platelets, without labeling megakaryocytes. The labeling is thought to be homogeneous in that all platelets are labeled equally. Labeling is also thought to be irreversible, without reutilization of the isotope after death of the cell. Both isotopes are capable of labeling platelets in vitro; these platelets are then reinjected into the host or recipient. DFP^{32} has also been utilized for in vivo labeling by intravenous injection of isotope directly into the host. With either isotope, platelets are isolated from the other formed elements at various time intervals and their radioactivity per number of platelets plotted against time. If arithmetic Y and X axes are used, a theoretical senescent lifespan should give a straight line (2–10). Conversely, a random hit survival curve should give an exponential curve (1, 12–14). Both types of curves have been reported.

Most investigators believe that the major component of the curve is linear, with probable "exponential" tailing toward the end of the survival (11, 15). Since methodology is so important in the derivation of these types of curves, it is convenient to explain this tailing as reutilization of isotope. A third type of platelet survival curve utilizes the amino acid analogue of methionine, Se^{75}-selenomethionine (16, 17). This isotope is purported to label a cohort population of young platelets. The isotope, which is injected in vivo,

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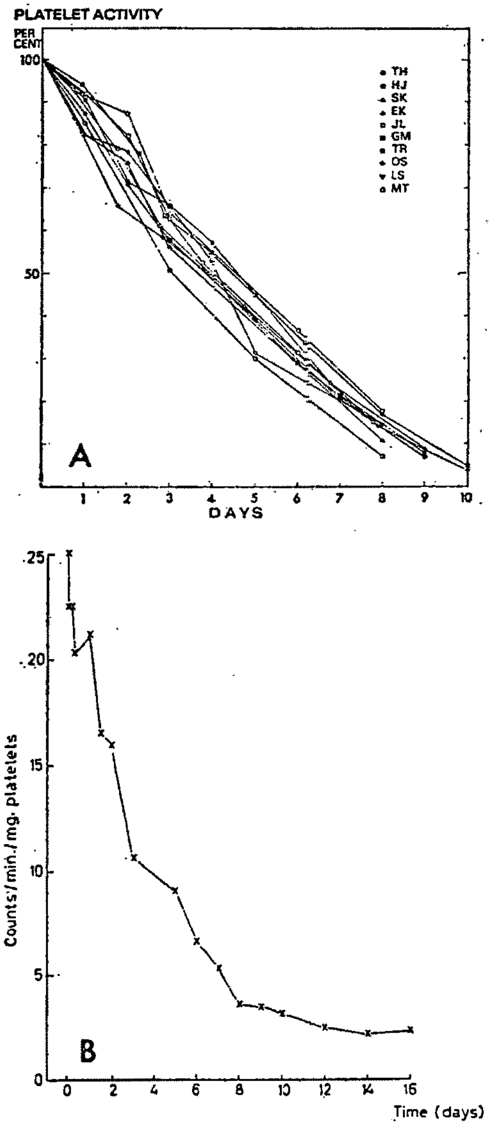

FIGURE 1A. Survival of Cr^{51}-platelets in 10 normal subjects. Kotilainen (9).

FIGURE 1B. An example of a DFP^{32} platelet survival curve in which a large number of points were obtained over the initial period and each sample was counted for 10,000 counts. Mustard et al (14).

does not label circulating platelets but does label megakaryocytes. A critical concentration of injected isotope leads to its complete removal from the circulation by platelets and other protein-synthesizing tissue, or by excretion. The isotope is also thought to be an irreversible label (although some reutilization does seem to take place). These properties make it possible to follow a platelet from birth to death. A theoretical senescent curve should provide an ascending limb, a plateau, and a descending limb. The approximation obtained in vivo may be seen in Figure 2 (dark circles). The time interval between the 50 percent ascending limb and the 50 percent descending limb is equivalent to the platelet survival, or approximately 10–12 days.

The above kinetic data provide strong support for the concept of human platelet senescence, i.e., birth of an early cell with a finite life span which is removed from the circulation at the termination of this life span. The following arguments support this thesis and will be presented below: 1. biochemical heterogeneity; 2. functional heterogeneity; 3. biochemical differences noted for experimentally produced "young" and "old" platelets; 4. functional differences noted for experimentally produced "young" and "old" platelets; 5. kinetic data supporting the isolation of a young platelet population which progresses with age to an older platelet population; 6. clinical data supporting the isolation of a young platelet population; 7. biochemical data supporting the transition of certain definable young platelets to older platelets.

BIOCHEMICAL HETEROGENEITY

If young platelets become older platelets that are removed from the circulation because they are old, certain properties of young and old platelets should be detectable which readily distinguish one from the other. The existence of biochemical heterogeneity would support such a thesis.

Human platelets can be separated by density into heavy and light populations by differential centrifugation in relatively inert specific density media (18). Thus, platelets remaining on the top of an oil with a sp. gr. of 1.030 have a density <1.030, whereas platelets sedimenting to the bottom of an oil with a sp. gr. of 1.040 have a density >1.040. The average volume of the heavy platelet population so isolated (as determined from the packed cell volume divided by the number of platelets per packed cell volume) is approximately 2-2.4-fold that of the light population. The heavy-large, light-small, and total platelet populations have been examined for biochemical heterogeneity, and data expressed per gram wet weight (rather than per platelet).

Glycogen, glycogenolysis, and glycolysis.—As can be noted from Table 1, heavy platelets contain more glycogen, orthophosphate, and adenine nucleotide (sum of ATP+ADP+AMP) than do light platelets (18). If these data were to be expressed per platelet, differences would be 2-2.4-fold greater since this is the difference in average volume between the 2 populations.

Heavy platelets have a glycolytic rate which is 4.2-fold greater than light platelets in the absence of glucose (Fig. 3). This rate probably represents glycogenolysis and can be attributed to the 3.4-fold greater glycogen content of heavy platelets compared to light platelets (18). It cannot be attributed to a decrease in platelet phosphorylase which is the same in both platelet populations (19) (Table 2). In the presence of glucose, heavy platelets have a glycolytic rate which is 2.6-fold greater than light platelets (18). This is consistent with the 2-fold higher enzyme content of heavy platelets

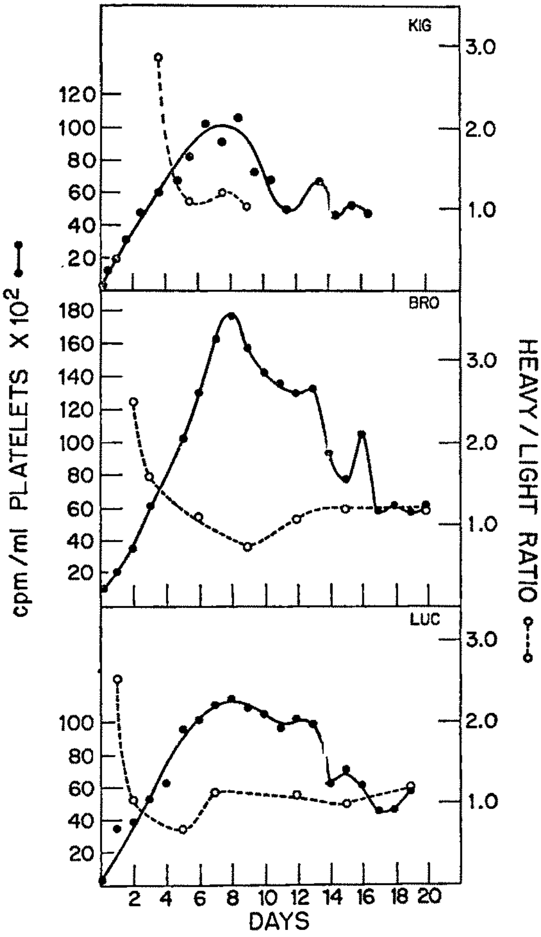

FIGURE 2. In vivo Se^{75}-selenomethionine platelet survival curves on subjects Kig., Bro., and Luc. Data are expressed as CPM/ml packed platelets (●). At varying time intervals, one unit of platelet-rich plasma was removed from the subject, processed into "heavy" and "light" platelet populations, and assayed for radioactivity and PCV. Data are expressed as above. The heavy/light ratio is plotted as (○). Amorosi, Garg & Karpatkin (16).

TABLE 1. GLYCOGEN, ORTHOPHOSPHATE AND ADENINE NUCLEOTIDE IN DIFFERENT PLATELET POPULATIONS[a]

	Light	Total	Heavy	H/L Ratio
Glycogen	10.7	27.6	36.7	3.43
Pi	2.08	1.59	2.68	1.3
ATP	1.78	2.70	3.90	2.2
ADP	2.01	1.79	1.89	0.94
AMP	0.410	0.495	0.633	1.5

[a] μmoles/gm wet weight.

for 6 of the 11 Embden-Meyerhof pathway enzymes: hexokinase, phosphohexoisomerase, phosphofructokinase, glyceraldehyde-3-P-dehydrogenase, phosphoglycerokinase and lactic dehydrogenase are in this group (Karpatkin and Strick, unpublished data).

Glycogenesis (incorporation of glucose-C^{14} into platelet glycogen) and glycogen synthetase activity.—When separate platelet populations were incubated in the presence of 5 m*M* glucose-C^{14} for 1 hr at 37°C, considerable differences were noted with respect to the incorporation of glucose into glycogen (Table 3). Thus, heavy platelets synthesized 5.9 times more glycogen than did light platelets per gram wet weight. However, since glycogen synthesis is dependent upon the presence of residual glycogen primer, and light platelets con-

TABLE 2. ENZYMES OF GLYCOGENOLYTIC AND GLYCOLYTIC PATHWAY IN DIFFERENT PLATELET POPULATIONS

Enzyme[a]	Light	Total	Heavy	Ratio
Phosphorylase				
(−AMP) Phos. a	11.2	13.0	13.0	1.2
(+AMP) Phos. (a&b)	36.6	39.9	40.6	1.1
Hexokinase	128	200	239	1.9
Phosphohexoisomerase	2,310	3,202	4,577	2.1
Phosphofructokinase	411	552	801	2.0
Aldolase	1,008	913	1,008	1.0
Triose-P-isomerase	7,860	8,152	7,860	1.0
Glyceraldehyde-3-P-dehydrogenase	357	499	709	2.0
Phosphoglycerokinase	894	1,592	1,619	1.8
Phosphoglyceromutase	688	733	708	1.0
Enolase	6,560	6,230	6,410	1.0
Pyruvate kinase	5,510	4,019	5,568	1.0
Lactic dehydrogenase	3,010	4,397	6,260	2.1

[a] μmoles substrate converted per gram wet weight per hour at 30°C.

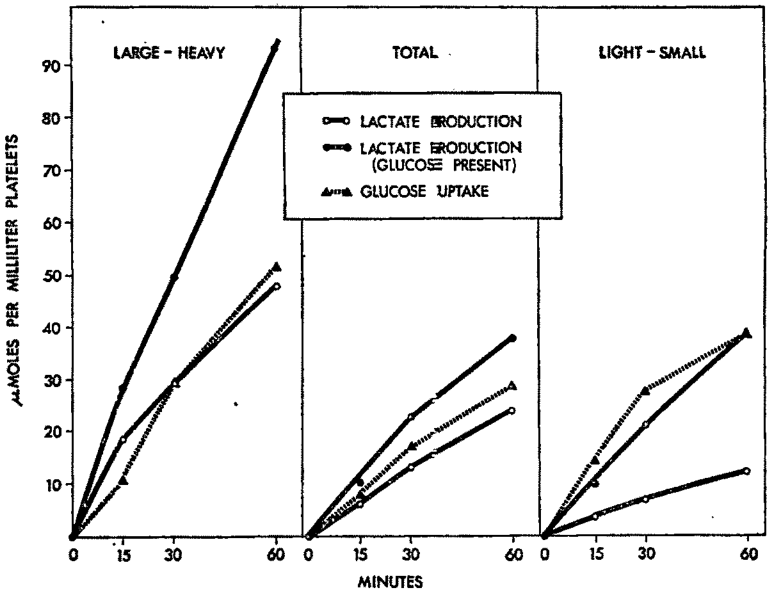

FIGURE 3. Lactate production and glucose uptake of separate platelet populations. Human platelets were separated into extreme density populations and incubated at 37°C for 15, 30, and 60 min. Data are expressed as μmoles/ml packed platelets. S.E.M. was less than 10%. Karpatkin (18).

tain less glycogen, a more meaningful measurement would be glycogen synthesized per μmole glycogen. This was 1.7-fold greater in heavy platelets. The increase in glycogen synthesis of heavy platelets was consistent with a similar increase in glycogen synthetase activity of 1.8-fold (19) (Table 4).

Glyconeogenesis (incorporation of pyruvate-C^{14} or citrate-C^{14}-into platelet glycogen) and fructose-1,6-diphosphatase activity.—Heavy platelets incorporated 3.9-fold more pyruvate into glycogen per gram weight wet, compared to light platelets and 1.7-fold more when data were expressed per

TABLE 3. GLYCOGENESIS, INCORPORATION OF GLUCOSE-C^{14} INTO GLYCOGEN OF DIFFERENT PLATELET POPULATIONS[a]

	Light	Total	Heavy	H/L Ratio
Glycogen μm/g wet weight	10.7	27.6	36.7	3.4
CPM/g wet weight	495	2231	2909	5.9
CPM/μm glycogen	46.4	80.7	79.3	1.7

[a] A 5% platelet suspension was incubated with 5 m*M* glucose-C^{14} for 1 hr at 37°C.

TABLE 4. GLYCOGEN SYNTHETASE AND FRUCTOSE-1, 6-DIPHOSPHATASE ACTIVITY[a] OF DIFFERENT PLATELET POPULATIONS

	Light	Total	Heavy	H/L Ratio
Glycogen synthetase				
(−G−6−P) Independent (I)	1.65	2.73	2.91	1.8
(+G−6−P) Total (D+I)	22.7	29.5	38.1	1.7
Fructose-1,6-diphosphatase				
(−AMP,−ADP)	4.70	7.21	11.3	2.4
(+AMP,+ADP)	9.48	14.5	24.5	2.6

[a] μm substrate converted/g wet weight/hour at 30°C for fructose-1,6-diphosphatase and 37°C for glycogen synthetase.

μmole glycogen (Table 5). Similar results were obtained with citrate incorporated into glycogen. These results were consistent with the 2.5-fold greater fructose-1,6-diphosphatase activity for heavy platelets (19) (Table 4).

Reductive capacity and resistance to lipid peroxidation.—Heavy platelets contain 1.9-fold greater GSH, 1.7-fold greater DPNH, and 1.2-fold greater TPNH than do light platelets. Light platelets contain 1.8-fold greater lipid peroxidation products than heavy platelets and are 2.4-fold more resistant to lipid peroxidation catalyzed by 0.1 m*M* $FeCl_3$, (Table 6) (Karpatkin and Strick, unpublished data).

Protein synthesis (incorporation of leucine-C^{14} into a platelet protein extract).—Although the protein concentration of heavy platelets was only

TABLE 5. GLYCONEOGENESIS, INCORPORATION OF PYRUVATE-C^{14} AND CITRATE-C^{14} INTO GLYCOGEN OF DIFFERENT PLATELET POPULATIONS

	Light	Total	Heavy	H/L Ratio
Pyruvate[a]				
nanomoles/g wet weight	31.1	86.0	121.3	3.9
nanomoles/μm glycogen	4.04	6.13	6.71	1.7
Citrate[b]				
nanomoles/g wet weight	21.7	34.7	64.3	3.0
nanomoles/μm glucose	2.64	2.71	3.80	1.4

[a] A 5% platelet suspension was incubated with 20 m*M* pyruvate-C^{14} at 37°C for 1 hr.

[b] Similar incubation as pyruvate, only 40 m*M* citrate was employed.

slightly greater than that of light platelets protein synthesis of heavy platelets was 3-fold greater per gram wet weight (18) (Table 7). Booyse, Hoveke & Rafelson (20), employing a sucrose gradient to separate heavy from light platelets, noted 50 times more protein synthesis in their heavy fraction compared to their light fraction. They concluded that only a small percentage of the total platelet population constitutes platelets that are highly active in protein synthesis. These workers also claimed to be able to isolate ribosomes from their heaviest fraction but not from the other fractions.

RNA content.—Ingram & Coopersmith (21) have presented histological evidence for increased RNA content of the large-heavy dog platelets derived following acute blood loss.

RESISTANCE TO OSMOTIC STRESS

Weber & Firkin (22) noted two morphologically identifiable populations of platelets following the exposure of human platelets to osmotic stress: either they appeared pale, round and slightly expanded, frequently showing

TABLE 6. REDUCTIVE CAPACITY AND RESISTANCE TO LIPID PEROXIDATION OF DIFFERENT PLATELET POPULATIONS

		Heavy	Total	Light	Heavy/Light[b] Ratio	Heavy[c] Incubation
GSH	(5)[a]	10.2	7.20	5.42	1.9	6.29
DPNH	(4)	0.049	0.035	0.029	1.7	0.036
TPNH	(4)	0.069	0.063	0.057	1.2	0.062
					Light/Heavy[e] Ratio	
Lipid perox.	(4)[d]	2.19		3.95	1.8	3.85
$+FeCl_3$	(5)	5.39		12.94	2.4	

[a] μm/g wet weight of initially extracted GSH, DPNH and TPNH of various platelet populations; number of experiments given in parentheses.

[b] Probability of Heavy/Light ratio being greater than 1 was significant at the $P<0.01$ level. S.E.M. was less than 10%.

[c] Heavy platelets were suspended in plasma (0.5 vol.%) and incubated for 1 hr (GSH) or 17 hr (DPNH, TPNH, Lipid peroxidation) at 37°C, prior to extraction.

[d] Nanomoles/gm wet weight of malonyl dialydehyde equivalents. Washed platelets were initially extracted; or suspended in human Ringer solution (0.5 vol.%) containing 0.1 m*M* $FeCl_3$ and incubated for 1 hr at 37°C prior to extraction; number of experiments given in parentheses.

[e] Probability of Light/Heavy ratio being greater than 1 was significant at the $P<0.01$ level. S.E.M. was less than 10%.

TABLE 7. Protein Synthesis of Different Platelet Populations[a]

	Light	Total	Heavy	H/L Ratio
Protein	103	105	115	1.1
CPM/g wet weight	3048	6265	9166	3.0
CPM/mg protein	29.5	59.7	80.0	2.7

[a] A 5% platelet suspension was incubated with 2 m*M* leucine-C^{14} for 1 hr at 37°C.

breaks in the cell membrane with apparent loss of cytoplasmic organelles; or they became darker and slightly contracted, having a continuous cell membrane with the usual cytoplasmic organelles. The authors postulated these observations reflected biochemical heterogeneity of the platelet population, and could have been due to platelet age of two distinct populations of platelets. Similar observations have been made with heavy and light platelet populations (Fig. 4). Heavy platelets were considerably more resistant to osmotic stress, with respect to ATP, ADP, and protein release into the media (18).

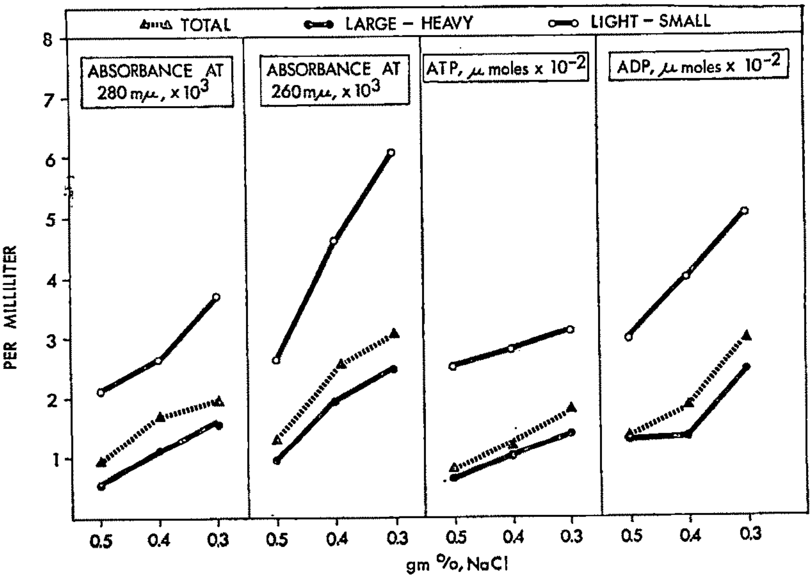

FIGURE 4. Resistance of separate platelet populations to osmotic shock. A 5% platelet suspension was exposed to 0.3, 0.4, and 0.5 g/100 ml NaCl containing 5 m*M* glucose for 5 min at 37°C. Release of material into the extracellular solution was measured with respect to 280 and 260 mμ absorbance and with respect to ATP and ADP release. Data are expressed per ml of extracellular fluid. Karpatkin (18).

TABLE 8. Effect of ADP Addition on ATP Release and ADP Consumption of Different Platelet Populations[a]

	Light	Total	Heavy	H/L Ratio	H/L Ratio
ATP Release	0.36	0.80	1.55	4.3	
ADP Consumption	1.85	1.35	0.30		6.2
Aggreg. time (min)	1.5	1.0	0.5		3.0

[a] A 5% platelet suspension was incubated in the presence of 5 mM glucose +0.1 mM ADP+2.5 mM $CaCl_2$ for 15 min at 37°C. Values are given as µm/g wet weight platelets.

FUNCTIONAL HETEROGENEITY

Platelet function was examined with respect to aggregation and the release reaction following the addition of ADP, thrombin, or epinephrine (23) (Tables 8–10). Heavy platelets aggregated 3.0-, 4.4-, and 3.3-fold more rapidly than did light platelets, respectively. Similarly, heavy platelets released 4.3-, 7.6-, and 6.4-fold greater ATP, respectively. After the addition of ADP, light platelets consumed 6.2-fold greater ADP than did heavy platelets. After addition of thrombin or epinephrine, heavy platelets released 6.0- and 4.0-fold greater ADP than did light platelets. Release of the antiheparin agent PF-4 was also examined following aggregation by ADP, thrombin, or epinephrine. This was 9.1-, 8.5-, and 12.4-fold greater for heavy platelets compared to light platelets.

Mannucci & Sharp (24) have made some very interesting observations with regard to platelet function and platelet size. They noted that platelet aggregation was associated with the selective aggregation of large platelets with all aggregating agents employed: ADP, epinephrine, thrombin, collagen, and 5-OH tryptamine, leaving the smaller platelets in suspension (Fig. 5).

TABLE 9. Effect of Thrombin and Epinephrine on ATP and ADP Release of Different Platelet Populations[a]

	Light	Total	Heavy	H/L Ratio	H/L Ratio
Thrombin (ATP)	0.18	0.95	1.36	7.6	
Thrombin (ADP)	0.26	0.88	1.56	6.0	
Aggreg. time (min)	6.2	1.8	1.4		4.4
Epinephrine (ATP)	0.18	0.71	1.15	6.4	
Epinephrine (ADP)	0.26	0.51	1.03	4.0	
Aggreg. time (min)	12.7	8.5	3.8		3.3

[a] Incubation same as Table 8; epinephrine 0.1 mM+1mM ascorbic acid; thrombin 1 NIH unit/ml.

TABLE 10. Effect of ADP, Thrombin, and Epinephrine on Release of Platelet Factor 4 From Different Populations

	Light	Total	Heavy	H/L Ratio
ADP	−1.2[a]	−10.9	−10.9	9.1
Thrombin	−1.2	−11.2	−10.2	8.5
Epinephrine	−0.7	−8.5	−8.9	12.7

[a] Numbers refer to decrement in clotting time from the control clotting time (i.e., shortening of the heparin-clotting time, for details see Ref. 23).

BIOCHEMICAL DIFFERENCES NOTED WITH EXPERIMENTALLY PRODUCED YOUNG AND OLDER PLATELETS

Protein synthesis.—Steiner & Baldini (25) have measured the incorporation of valine-C^{14} into TCA-precipitable protein of young rat platelets which have recently entered the peripheral blood following the induction of thrombocytopenia with anti-platelet antibody. Young platelets with an average life of 1–3 days were compared to old platelets with an average life of 3–6 days. An approximate 2-fold increase in protein synthesis was noted

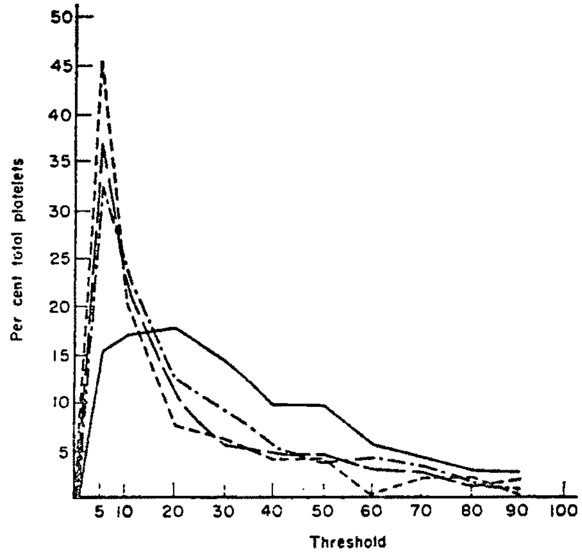

Figure 5. Size of unaggregated platelets remaining in suspension following ADP aggregation. ———, PRP+saline; -----, PRP+ADP, $6.5M \times 10^{-6}$; — — —, PRP+ADP, $6.5M \times 10^{-7}$; —·—·—, PRP+ADP, $6.5M \times 10^{-8}$, Mannucci & Sharp (24).

for young platelets compared to older ones. Shulman et al (26) examined protein synthesis (incorporation of leucine-C^{14} in new platelets obtained from idiopathic thrombocytopenic purpura patients after 2–3 days of treatment with 60 mg of prednisone, during which time their platelets rose from $<2000/mm^3$ to $>40,000/mm^3$. This was compared to platelets from hematologically normal individuals given the same amount of prednisone. The regenerating younger platelets of ITP patients synthesized approximately four times as much protein, as CPM/mg protein, as did normal platelets.

Lipid peroxidation.—Okuma, Steiner & Baldini (27) examined lipid peroxidation products in young versus old rat platelets immunologically produced as in their protein synthesis studies. They examined lipid peroxidation products as well as resistance to lipid peroxidation following catalysis by $FeCl_3$. Six-day-old platelets had 1.7-fold more lipid peroxidation products than 2-day-old platelets. Following activation of lipid peroxidation with $FeCl_3$, 6-day-old platelets had 3-fold greater lipid peroxidation products compared to 2-day-old platelets (Table 11).

ATP, phospholipid, cholesterol, protein, and RNA content.—Ginsburg & Aster (28) harvested young rat platelets following induction of thrombocytopenia with either anti-platelet antibody or exchange transfusion with platelet-poor blood. Old rat platelets were produced by harvesting platelets

TABLE 11. LIPID PEROXIDE VALUES IN RAT PLATELETS

Time After Injection of Antiserum (days)	Lipid Peroxides[a] (nanomoles malonaldehyde/1×10⁹ platelets)	
	Control	+$FeCl_3$
0	0.38±0.03	3.17±0.27
2	0.22[b]	1.05[b]
3	0.25±0.02	2.05±0.25
4	0.27±0.04	2.66±0.31
5	0.36±0.03	2.98±0.30
6	0.37±0.04	3.20±0.29

[a] Each value was obtained from 5 rats (Mean±1 S.D.).

[b] Values were obtained from platelets pooled from 5 animals.

Control refers to platelets suspended in 0.2 *M* trismaleate buffer (pH 5.9) without addition of metal salt. Lipid peroxides were measured without delay in this group.

+$FeCl_3$ refers to platelets suspended in the same buffer as the control groups but with addition of $FeCl_3$ ($1\times10^{-4}M$). Lipid peroxides were measured following 1 hr incubation at 37°C. In both groups each determination was done in duplicate.

Ref. 27, Okuma, Steiner & Baldini.

TABLE 12. Platelet Radioactivity and Platelet Count before and after Exposure of ^{35}S-Labeled Platelets to Collagen Fibers in EDTA Platelet-Rich Plasma

Experiment	Platelet radioactivity		Platelet count	
	Before collagen	After collagen	Before collagen	After collagen
	cpm/10^6 platelets		*No./mm^3 × 10^3*	
54 hr after $^{35}SO_4^-$ injection				
1	98	30	240	209
2	111	74	266	214
3	114	111	270	284
4	74	37	348	314
5	190	100	290	210
6	188	134	300	285
7	107	44	320	210
8	348	340	261	241
9	238	139	304	264
10	269	218	265	260
Significance of differences between values before and after exposure to collagen	$t=5.19$ $P<0.001$		$t=3.25$ $P<0.01$	
96 hr after $^{35}SO_4^-$ injection				
1	21	39	176	145
2	45	44	432	360
3	101	100	290	250
4	16	16	276	280
5	36	37	258	230
6	443	375	397	340
Significance of differences between values before and after exposure to collagen	$t=0.65$ $P<0.6$		$t=4.33$ $P<0.01$	

Ref. 29, Hirsch, Glynn & Mustard.

6 days post-irradiation of the bone marrow. These authors noted a 33 percent higher ATP content, 30 percent higher phospholipid content, and 10 percent higher cholesterol and protein content in young rat platelets (0–3 days old) compared to older rat platelets.

FUNCTIONAL DIFFERENCES NOTED WITH EXPERIMENTALLY PRODUCED YOUNG AND OLD PLATELETS

Hirsch, Glynn & Mustard (29), employing rabbit platelets, noted a greater adherence of "physiologically" young platelets to collagen. These authors used an ingenious technique for measuring platelet function in physiologically young or old rabbit platelets. Rabbits were injected with either $S^{35}O_4$ or glycine-C^{14}. Their EDTA-anticoagulated platelet-rich plasma (PRP) was removed at various days post-injection of isotope and incubated in vitro with collagen. The CPM/platelets or specific activity of the PRP was then determined before and after exposure to collagen. A fall in specific activity of PRP was noted in 2–3-day-old platelets, but not in 4-day-old platelets (Table 12). In another experiment, animals were continuously injected with isotope for 7 days in order to obtain a high specific activity, and platelets were then harvested 72 hr after the last injection. Under these conditions, an old platelet population was harvested. When these platelets were reacted with collagen, the specific activity of the residual PRP increased (Table 13).

Shulman et al (26) have provided evidence that only young platelets are hemostatically effective and that these are normally sequestered by the spleen. They, as well as others, have noted that a bleeding time of greater than 10 min may occur with a platelet count of 100,000/mm^3; or, conversely, one can have a normal bleeding time with a platelet count of 15,000/mm^3. In Figure 6 the platelet count is plotted following recovery from quinidine-

TABLE 13. Platelet Radioactivity and Platelet Count before and after Exposure to Collagen Fibers of a Population of Platelets Containing ^{35}S-Labeled Old Platelets in EDTA-PRP

Experiment	Platelet radioactivity		Platelet count	
	Before collagen	After collagen	Before collagen	After collagen
	cpm/10^6 platelets		*No./mm^3 × 10^3*	
1	42	83	276	204
2	70	72	284	264
3	69	72		
4	218	229	290	215
5	445	531	225	189
6	600	630	223	178
7	104	111	250	224
8	99	110	260	240
9	102	112	190	160
Significance of differences between values before and after exposure to collagen	$t=2.44$ $P<0.05$		$t=5.57$ $P<0.005$	

Ref. 29, Hirsch, Glynn & Mustard.

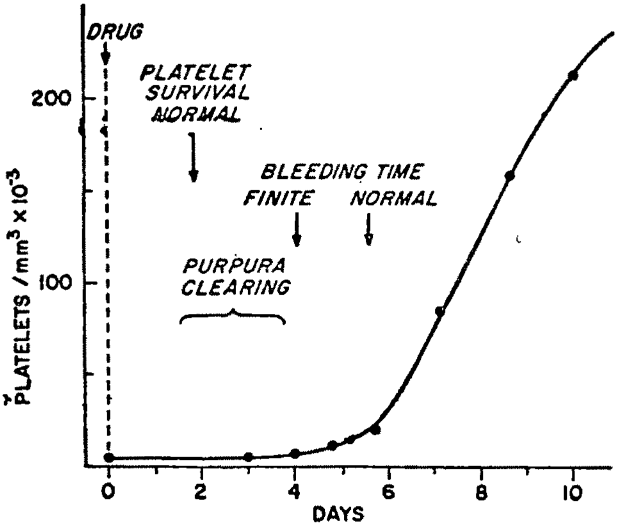

FIGURE 6. Correlation between bleeding time and platelet level during recovery from severe quinidine purpura. Shulman et al (26).

induced thrombocytopenia. To be noted is the abnormal bleeding time at 4 days (platelet count approximately 10,000/mm^3) and the normal bleeding time at 5-3/4 days (platelet count approximately 20,000/mm^3) presumably owing to young platelets. Conversely, transfusion of platelets obtained from a normal donor (Fig. 7) gave a prolonged bleeding time of 10 minutes, despite a platelet count of 300,000/mm^3; whereas a transfusion of young platelets from an asplenic donor gave a prolonged bleeding time of 10 min only when the platelet count dipped to approximately 50,000/mm^3. These authors also noted better clot retraction in recipients of asplenic donors compared to normal donors.

Johnson et al (30) have reported platelet function data from a unique patient with thrombopoietin deficiency. This 18-year-old female has chronic thrombocytopenia which responds to a transfusion of fresh frozen plasma, rising from chronic levels of 10,000/mm^3 to levels of 200–300,000/mm^3 on day 4 and to a peak of 500–800,000/mm^3 on day 9–10, and gradually returning to 10,000/mm^3 by day 21–23. Following infusion of the plasma factor, young platelets were harvested from this patient on day 4 and old platelets on day 22. The bleeding time was significantly prolonged in "old" platelets. Platelet adhesiveness tests performed by 3 different methods (including an in vivo technique) revealed increased adhesiveness in young platelets and decreased adhesiveness in old platelets. Platelet factor 3 activity of young platelets was significantly greater than that of old platelets.

The above biochemical and functional similarities between young platelets isolated experimentally and density-separated, large-heavy platelets strongly suggest that they may be identical.

KINETIC DATA SUPPORTING THE ISOLATION OF A YOUNG PLATELET POPULATION

McDonald, Odell & Gosslee (31) were the first to propose that young platelets were large platelets which progressed with age to smaller platelets. These investigators produced young platelets from rats recovering from thrombocytopenia induced with anti-platelet antibody. Old platelets were prepared by transfusing normal platelets into rats made platelet-deficient

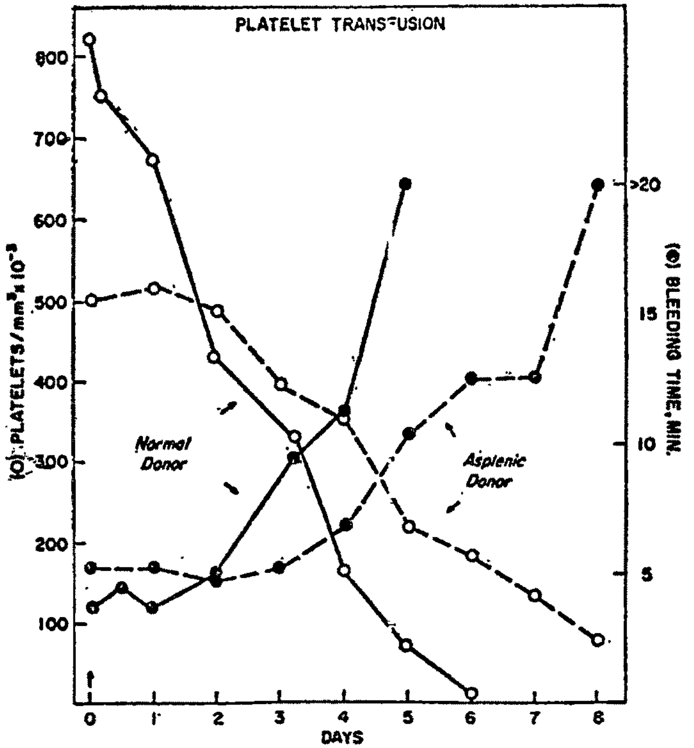

FIGURE 7. Survival of transfused platelets obtained from normal and splenectomized donors, and their effect on the bleeding time. Recipients were small children with severe thrombocytopenia owing to aplastic anemia, leukemia, and Aldrich syndrome, who required platelets therapeutically. One splenectomized donor had his spleen removed following traumatic rupture. Two other donors had had splenectomy for ITP more than 10 years prior to donating, and had normal platelet counts and platelet survival. Shulman et al (26).

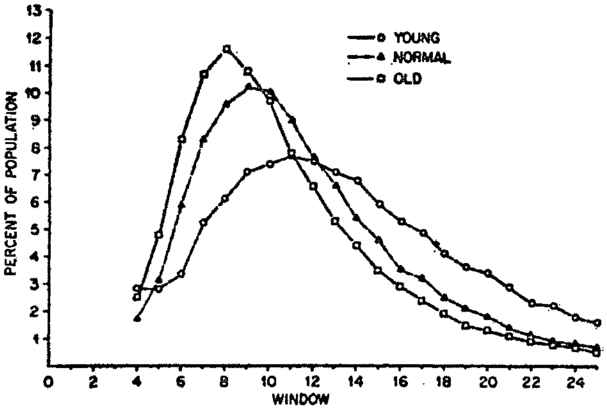

FIGURE 8. Size distributions of platelets of young, normal, and old populations. Curves of young and old platelets are the means of 3 rats, and of normal platelets, 2 rats. Size is shown as window number rather than in μ^3. McDonald et al (31).

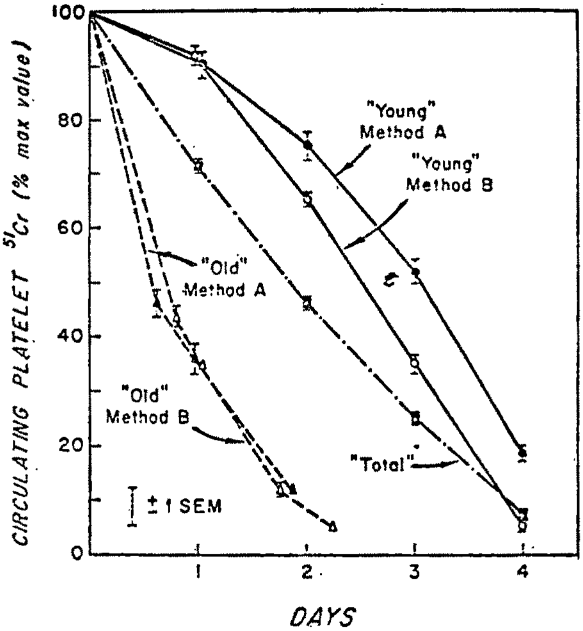

FIGURE 9. Survival patterns of "young," "total," and "old" platelet populations. Points represent mean values ± one standard error of the mean. Ginsburg, A. D. 1969. *J. Lab. Clin. Med.* 174:141

with 900 r of whole body irradiation, and re-collecting them two days later. Platelet volume was measured with a Coulter Electronic Particle Counter, and size distribution curves are shown in Figure 8.

Minter & Ingram (32) have noted that following the thrombopoietic stimulus of acute blood loss in the dog, platelet size and volume increase.

Ginsburg & Aster (28) have noted that in the rat old platelets are diminished in volume by 22 percent. These investigators (28), employing Cr^{51}-labeled cohorts of young and old platelets (see above for methodology), concluded that senescence is the major factor governing removal of platelets from the circulation (Fig. 9). While the platelet survival of the total population was nearly linear, old cells had both an exponential and strikingly shortened survival, and young cells were removed more slowly from the circulation.

Booyse, Hoveke & Rafelson (20), employing sucrose gradient techniques, noted a larger percentage of heavy platelets following recovery from thrombocytopenia in the patient of Johnson et al (30), and a smaller percentage of heavy platelets in a patient with drug-induced marrow suppression.

Amorosi, Garg & Karpatkin (16) employed Se^{75}-selenomethionine studies to differentiate young from older platelets (Fig. 2). Platelets were harvested at various time intervals during the platelet survival and separated into heavy-large and light-small populations. An increase in the heavy/light ratio on early days of the survival, followed by a decrease in this ratio on late

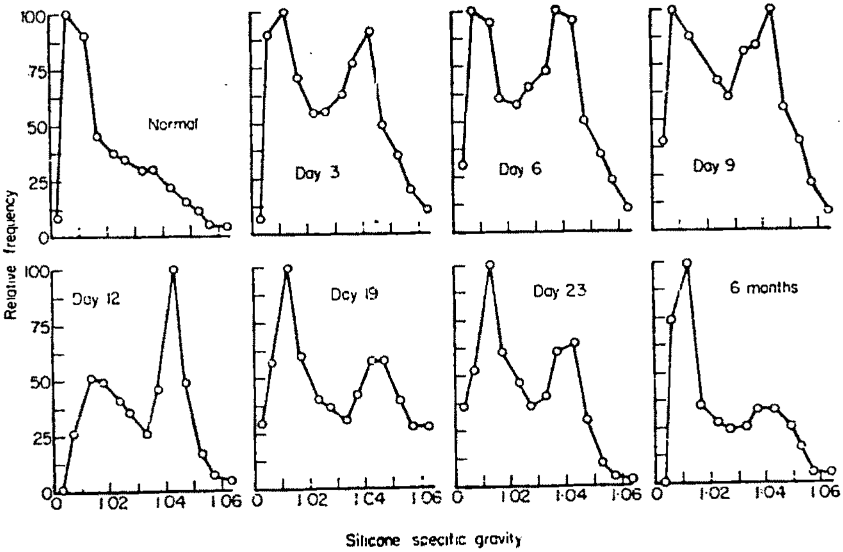

FIGURE 10. Sequential changes in the platelet density histogram following blood loss. In dogs, a population of high-density platelets appeared on the third day after the first bleeding. Two distinct populations could still be identified on the 23rd day.

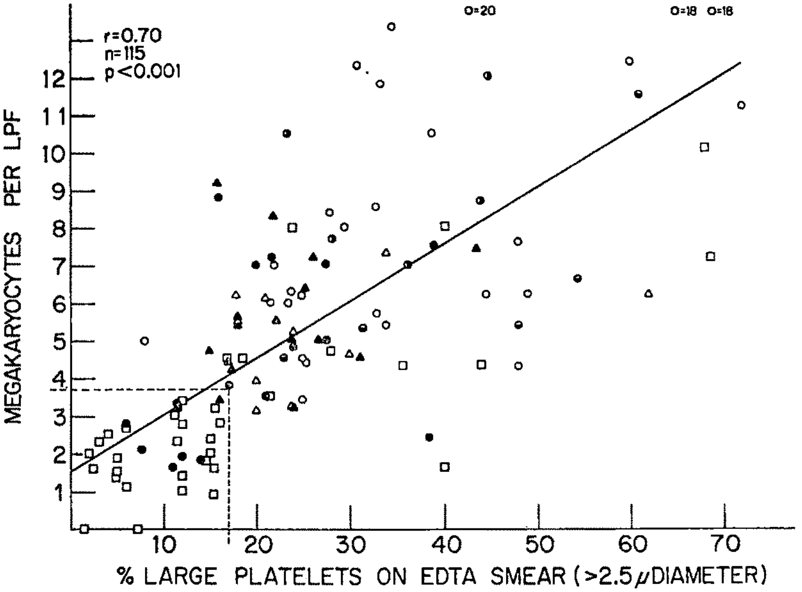

FIGURE 11. Correlation between % of megakaryocytes per low power field (100×) The open circles refer to ITP, the close circles to SLE, the horizontal half-closed circles to DIC, the vertical half-closed circles to drug-induced thrombocytopenia, the open triangles to hypersplenism, the closed triangles to iron-deficiency anemia (normal or low platelet count), and the open boxes to other disorders. The interrupted lines include +2 S.D. of the "control mean."

days during the survival, is consistent with the concept that heavy-large platelets are young platelets which progress with age to smaller-lighter platelets. Similar conclusions were drawn by Karpatkin, employing DFP^{32}-labeled rabbit platelets (18).

The above kinetic considerations indicate that heavy-large platelets are young platelets and suggest that these become lighter-smaller platelets with age. However, the data do not indicate that large-heavy platelets are the only young platelets. Nor do they rule out the possibility that large-heavy platelets might be "stress" platelets with a shortened platelet survival, which are produced secondary to the induction of thrombocytopenia. Indeed, Minter & Ingram (32) have proposed that these large-heavy platelets obtained from acutely bled dogs are young stress platelets with a normal life span (Fig. 10). However, this conclusion is not supported by the data of Amorosi, Garg & Karpatkin (Fig. 2) which were obtained from "basal" control patients who were not thrombocytopenic and who were not stressed (thrombopoietically) during the early time points of the experiment. It is conceivable that two varieties of large-heavy platelets exist: one that is physiologic with a normal life span; the other that is pathophysiologic, or "stress-released," with either a normal or shortened life span.

CLINICAL DATA SUPPORTING THE ISOLATION OF A YOUNG PLATELET POPULATION

Regardless of whether large-heavy platelets are young "physiologic" platelets or young stress platelets, they may conveniently be used to predict megakaryocyte number (33), and hence platelet turnover. Thus, an excellent correlation exists between the percentage of megathrombocytes (large platelets) and the number of megakaryocytes in the bone marrow for all situations with normal or low platelet counts, except megaloblastic anemia ($r=0.70$, $P<0.001$, Fig. 11). In megaloblastic anemia, megathrombocytes are always present on peripheral smear although megakaryocyte number is often decreased.

The above data indicate the usefulness of the megathrombocyte as an index of megakaryocyte number (that is, thrombopoiesis) both in disorders of increased peripheral platelet destruction, such as ITP, drug-induced thrombocytopenia, disseminated intravascular coagulation (DIC) and systemic-lupus erythematosus (SLE); and in other disorders such as hypersplenism, aplastic anemia, and iron-deficiency anemia (without thrombocytosis). A summary for patients with various clinical disorders (33) is presented in Table 14. It is apparent that the average human bone marrow can increase its megakaryocyte number at least 4.4 times. The increase in megakaryocyte number is paralleled by an increase in the percentage of megathrombocytes.

TABLE 14. MEGATHROMBOCYTE AND MEGAKARYOCYTE ENUMERATION IN CLINICAL DISORDERS

	% Megathrombocytes	Megakaryocyte number	Number thrombocytopenic/total
CONTROL	10.8±3.1	2.1±0.8	
ITP	38.5 (3.6)[a]	9.3 (4.2)	22/26[b]
Drug-induced	35.3 (3.3)	9.2 (4.4)	5/5
DIC	35.9 (3.3)	5.9 (2.8)	6/6
SLE	28.6 (2.6)	4.8 (2.3)	5/5
SLE	19.1 (1.8)	4.4 (2.1)	5/17
Hypersplenism	27.1 (2.5)	5.1 (2.4)	10/10
Aplastic anemia	3.7 (0.35)	0.6 (0.29)	4/4
Megaloblastic anemia	36.9 (3.5)	1.9 (0.9)	14/16

[a] Number in parentheses refers to increase over control values.

[b] Two patients were in remission and 2 patients had easy bruising with probable ITP.

TABLE 15. Compensated Thrombocytolytic State
(Normal Platelet Count with Increased Megathrombocytes and Megakaryocytes)

Disease	Percent Megathrombocytes[a]	Megakaryocyte number[b]	Platelet survival, T1/2[c]	Anti-platelet Ab titer[d]
Controls	10.8±3.1(S.D.)	2.1±0.8(S.D.)	115 hrs.	0–1:4
Chronic ATP[e]	38.8	10.5	N.T.[f]	1:40
Chronic ATP	25.0	4.5	N.T.	1:4
Chronic ATP	25.0	6.2	66	1:16
Easy Bruising[g]	25.3	4.4	N.T.	1:32
Easy Bruising	54.0	11.8	42	1:48
"Normal" (R.S.)	26.7	N.T.	55	1:8
SLE	16.0	8.8	65	1:4
SLE	20.0	7.0	32	1:32
SLE	24.0	4.8	34	N.T.
SLE	16.0	N.T.	61	1:44
SLE	16.8	N.T.	71	N.T.
SLE	21.7	7.2	N.T.	1:12
SLE	27.6	5.0	N.T.	1:8
SLE	27.5	7.0	N.T.	N.T.

[a] % platelets on EDTA smear with a diameter greater than 2.5μ.

[b] Average number of megakaryocytes per spiculefield (low power, 100×) enumerated from bone aspiration smears examining a minimum of 25 fields.

[c] In vivo DFP^{32} platelet survival studies. The % megathrombocytes was measured at the beginning and end of the platelet survival study and remained elevated in the seven patients studied. The platelet counts remained constant and varied in different individuals between 210,000–267,000/mm^3.

[d] Platelet Factor 3 "immuno-injury" anti-platelet antibody titer expressed as the maximal dilution of globulin fraction of patient's serum giving positive test. Titers greater than 1:4 are considered significant.

[e] Chronic Autoimmune Thrombocytopenic Purpura "in remission," also referred to as ITP.

[f] Not tested.

[g] Probable compensated ATP with history of easy bruising and negative SLE preparations.

Compensated thrombocytolytic state.—A compensated thrombocytolytic state (normal platelet count with increased number of megakaryocytes and megathrombocytes) was found in eight patients with SLE, three with chronic ITP in remission, and two with a history of easy bruising (33, 34) (Table 15). Seven of these patients were further studied with in vivo DFP^{32} platelet-survival curves. All seven had an exponential decline in platelet radioactivity (random destruction) with a half-time of 32 to 71 hr (Fig. 12). This may be compared to linear decline in platelet radioactivity (senescent removal) with a half-time of 115 hr in seven healthy volunteer subjects and three patients with SLE who had normal platelet counts and normal percentages of megathromboyctes.

Karpatkin, Garg & Siskind (34) screened 105 healthy laboratory personnel (forty-six men and fifty-nine women, average age, twenty-nine years) for the number of megathrombocytes and platelet count. In this group, one subject (R.S.) was consistently above 2 S.D. from the mean (Fig. 13). A platelet survival curve in this healthy asymptomatic 60-year-old white woman with normal platelet count, hematocrit, white blood cell count and differential, was significantly shortened and exponential with a T 1/2 of 55 hr (Fig. 12). The patient had no detectable antinuclear antibody. The antiplatelet antibody titer in this subject was 1:8 (borderline positive), described in Table 15.

Disease categories were next examined where compensated thrombocytolytic states might be predicted (Garg, Lackner and Karpatkin, unpublished data).

1. Autoimmune destruction of platelets such as might be the case in chronic idiopathic thrombocytopenic purpura in remission (also referred to as chronic autoimmune thrombocytopenic purpura (ATP) (34, 35), or systemic lupus erythematosus.

2. Increased utilization of platelets such as might be the situation post-surgery, or in mild disseminated intravascular coagulation.

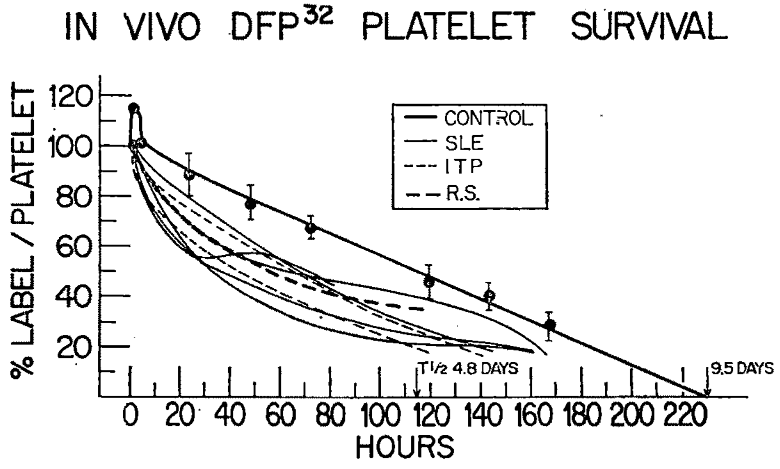

FIGURE 12. In vivo DFP^{32} platelet survival studies in ten control subjects compared with eight subjects having increased numbers of megathrombocytes and normal platelet counts.

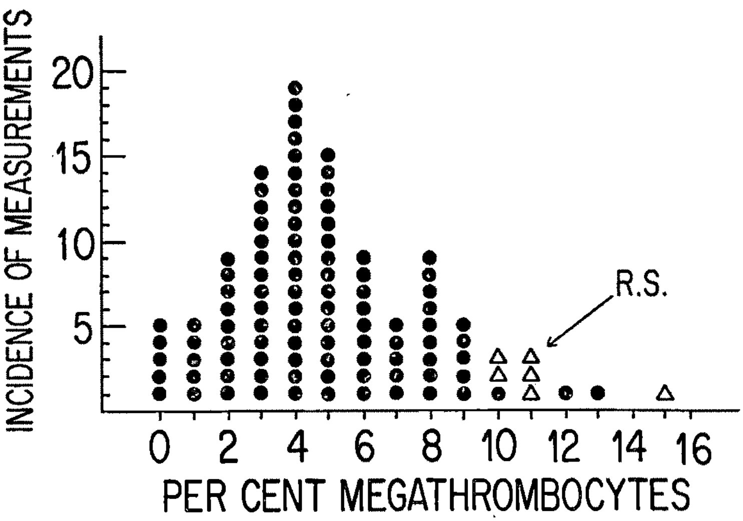

FIGURE 13. Percent megathrombocytes in 105 healthy laboratory personnel with normal platelet counts.

3. Increased mechanical destruction of platelets such as might be the situation in rheumatic heart disease with abnormal heart valves and/or prosthetic heart valves, or small vessel angiopathies as might be the situation in diabetes mellitus with retinopathy.

These data are shown in Figure 14, which indicates the percentage of megathrombocyte elevation (EDTA smear measurements) in these disorders. Significant elevations were noted in 72 percent of SLE patients, in 63 percent of patients with rheumatic heart disease and severe valvular involvement, in 55 percent of patients with chronic autoimmune thrombocytopenic purpura in remission, in 50 percent of patients with diabetes mellitus and retinopathy, and in 43 percent of patients with DIC.

BIOCHEMICAL DATA SUPPORTING THE TRANSITION OF LARGE-HEAVY (YOUNG) PLATELETS TO SMALLER-LIGHTER (OLDER) PLATELETS

When heavy platelets were incubated in their own plasma at 37°C for 17 hr, the group of 6 enzymes with twice the activity in heavy versus light platelets were the more labile ones (Fig. 15). These include the 3 rate-limiting enzymes, hexokinase, phosphofructokinase, and glyceraldehyde-3-P-dehydrogenase. The increased lability of this group of enzymes was attributed to oxidative stress, since reducing agents were capable of blocking the in vitro

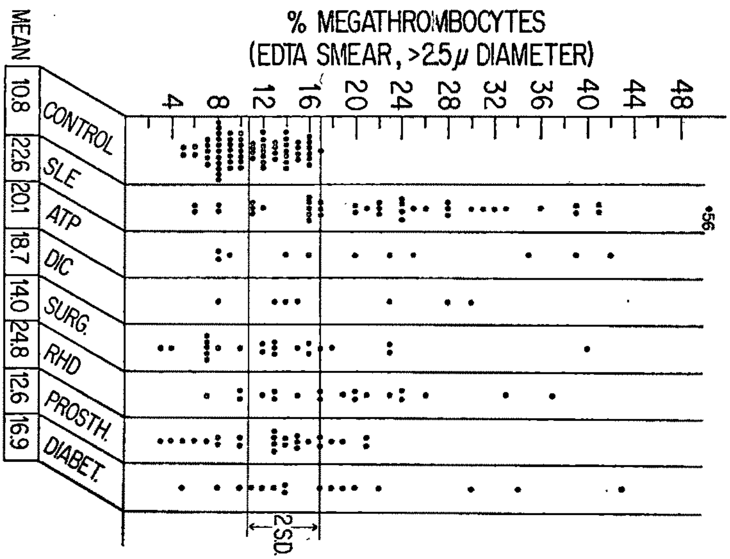

FIGURE 14. Scattergram of percent megathrombocytes (EDTA smears) in patients with normal platelet counts and varying clinical disorders. The lower horizontal line refers to the mean % megathrombocytes for 67 healthy laboratory personnel. The upper horizontal line represents +2 S.D. from the mean.

TABLE 16. EFFECT OF CORTISONE ACETATE, CHLOROQUIN HYDROCHLORIDE, DITHIOTHREITOL, ON IN VITRO ENZYME LABILITY OF HEAVY PLATELETS FOLLOWING INCUBATION AT 37°C FOR 17 HOURS

Enzymes	Control[a] (4)	Cortisone (2)	Chloroquin (1)	Dithiothreitol (3)
Hexokinase	73	58	64	123
Phosphohexoisomerase	75	63	64	109
Phosphofructokinase	69	57	58	126
Glyceraldehyde-3-P dehydrogenase	71	61	58	107
Phosphoglycerokinase	85	73	63	101
Lactic dehydrogenase	69	60	48	82

[a] Percent of initial enzyme activity following 17 hr incubation at 37°C in plasma, 100 units/ml penicillin, and additional 150 mg/ml glucose. Number of experiments is given in parentheses. Concentration of effector agents: cortisone acetate, $10^{-4}M$; chloroquin hydrochloride, $10^{-4}M$; dithiothreitol, $10^{-5}M$.

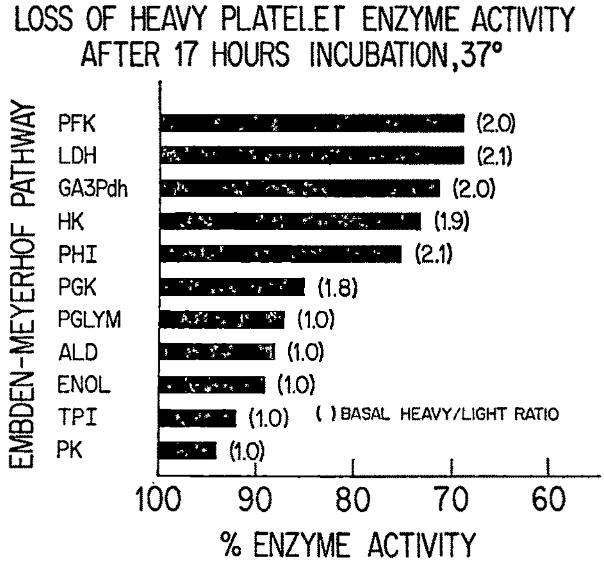

FIGURE 15. In vitro incubation of heavy platelets for 17 hr at 37°C. Empirical Vmax was measured at zero time and following 17 hr of sterile incubation in plasma with 0.5 vol. % platelet suspension. Percent enzyme activity is compared to initial heavy/light ratio.

lability [whereas lysosomal stabilizing agents were incapable of doing this (Table 16)]. The increased lability of this group of enzymes was associated with a decrease in GSH, DPNH and TPNH content, and an increase in lipid peroxidation products (Table 6).

These data indicate that heavy platelets are also more resistant to oxidative stress than are light platelets. The mechanism of the increased lability is apparently related to oxidative processes within the cell or on the membrane, since reducing agents can partially or completely reverse this effect in heavy platelets. This is consistent with the increased resistance to lipid peroxidation in heavy platelets. It is conceivable that free radical formation might be responsible for the oxidative changes noted during in vitro incubation of heavy platelets. Differences in enzyme activity might also be related to the greater reductive capacity and resistance to lipid peroxidation of heavy platelets compared to light platelets. Free radical formation is further supported by the apparent increase in lipid peroxidation following incubation of heavy platelets, associated with a decrease in enzyme activity. Lipid peroxides and their products have been shown to be metabolic poisons capable of forming inactivating complexes with proteins (36, 37) and essential enzymes (38–40). Oxidation of sulfhydryl groups (37, 39), microsomes, mitochondria (41–44) and swelling and lysis of mitochondria (44), have been reported secondary to lipid peroxidation. Indeed, free radical formation has been incriminated as one of the mechanisms responsible for the process of aging (45, 46).

If lability of rate-limiting glycolytic and other enzymes secondary to oxidative stress is associated with in vitro platelet senescence, it might also be associated with in vivo senescence. Indeed, increased resistance to lipid peroxidation has recently been reported in young rat platelets obtained following the administration of rabbit anti-rat platelet antibody (27). Furthermore, Murphy & Gardner (47) have recently presented evidence from platelet preservation studies that in vitro aging approximates in vivo aging. It should be emphasized, however, that the association of rate-limiting enzyme lability with in vitro incubation and oxidative stress does not prove a causal relationship.

However, if heavy-large young platelets do indeed become lighter-smaller older platelets as has been suggested (16, 18, 28, 31), it would be tempting to speculate that this could occur as a result of, or associated with, loss of glycolytic rate-limiting enzyme activity. This, in turn, would contribute to lower energy stores, i.e., glycogen and adenine nucleotide content. The platelet, being an aerobic glycolytic tissue, is dependent to a very large extent upon the Embden-Meyerhof pathway for its energy requirement (48–50). This energy is required for the maintenance of cationic gradients (51), maintenance of steady-state conditions (50), and maintenance of platelet discoid shape (50, 52). Consequently, any impairment in energy storage or utilization would be a decided disadvantage to the cell with respect to viability. Energy is also required for platelet functions such as ADP-induced platelet-plug formation (53), clot retraction (53), synthesis of phospholipid (54), active transport of amino acids (55) and serotonin (56).

CONCLUDING REMARKS

Platelet survival studies with both cohort and noncohort labels are compatible with theoretical platelet senescent curves. Biochemical and functional data have been described which indicate that human platelets are heterogeneous. The heavy-large platelet is a better equipped cell compared to the lighter-smaller cell in the biochemical sense, with respect to ability to generate ATP and maintain cationic gradients with the external milieu; and in the functional sense with respect to ability to maintain hemostasis. Kinetic data indicate that heavy-large platelets are young platelets, recently released from the marrow. These data do not preclude the possibility that heavy-large platelets may be stress platelets, nor that heavy-large platelets are the only young platelets. Large platelets (megathrombocytes) may be used as a convenient tool to predict megakaryocyte number, and to detect compensated thrombocytolytic states. Both kinetic and biochemical data suggest that large-heavy platelets become smaller-lighter platelets with age. The above considerations strongly support the existence of human platelet senescence.

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THERMAL CONTROL IN PREMATURE INFANTS 7033

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INTRODUCTION

There is now general agreement that the full size, intact newborn baby born at term is a homeotherm whose thermoregulatory range nevertheless is limited by adult standards. When challenged by heat or cold, the normal baby shows prompt, typical homeothermic responses designed to maintain deep body temperature near its set point. In response to heat, the baby shows vasodilation (particularly in the hands and feet), which favors increased rate of heat loss by radiation and convection. In addition, sweating occurs in association with rectal temperatures above 37.2°C, thus favoring increased rate of heat loss by evaporation. When challenged by cold, on the other hand, the normal baby attempts to conserve body heat by cutaneous vasoconstriction, and to maintain body temperature by increasing his rate of heat production. Although this latter response may involve muscular thermogenesis (including shivering if the cold stress is severe), the baby usually relies on nonshivering thermogenesis.

Babies born prematurely may also show the typical homeothermic responses indicated above, but many of them suffer by comparison with term infants with respect to both qualitative and quantitative aspects of thermoregulatory response. The small-for-date infant is worthy of separate consideration, moreover. While he suffers a limitation to his thermoregulatory range that is imposed by his small body size, he nevertheless may show comparatively well developed homeothermic responses insofar as these depend on gestational age.

Both categories of low birth weight infant—the preterm and the small-for-date infant—will be considered in this review.

HEAT PRODUCTION

Methods

Both direct (1) and indirect (2) calorimetry have been employed in the measurement of heat production of the newborn infant. The usual indirect approaches are: (*a*) determination of rate of oxygen consumption, sometimes in conjunction with measurement of carbon dioxide production and of uri-

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nary nitrogen excretion: (*b*) determination of insensible water loss. The caloric equivalent of the measured oxygen consumption, normally about 4.8 cal/liter, varies somewhat with the composition of the metabolic mixture, and can be estimated therefore by measuring urinary nitrogen excretion, oxygen consumption and carbon dioxide production (3). During the first week of postnatal life, systematic differences in respiratory quotient (RQ) have been described as functions of postnatal age, feeding policy, and thermal exposure. Since Hasselbalch (4), various authors (5–7) have confirmed a fall in RQ from close to one at birth to about .75 on the third day, thereafter slowly rising to .85 or above by day seven or eight. With the introduction of early feeding, the early decline of RQ is terminated at 12 hr of age, and RQ rises over the next few days (8). Small-for-date babies tend to have lower RQ's than normally grown ones (8). Cold exposure is associated with fall in RQ (9).

The caloric equivalent of the insensible water loss is 0.6 cal/g. Hey & Katz (10) have recently confirmed that newborn babies, including premature infants, dissipate a constant proportion of basal heat production—about 23 percent—by evaporation under basal circumstances.

Experimental conditions.—The metabolic rate of premature infants depends on activity, food, and temperature. It has long been appreciated that activity increases the metabolism in premature infants (11, 12), and, more recently, correlations between metabolic rate, activity, and sleep state have been described (13). A metabolic increase resulting from the ingestion of food has also been known for a long time. This increase has recently been carefully quantitated (14) as representing 9.2 percent of the ingested calories in low birth weight infants fed an artificial formula containing 3.4 percent protein. In this study, the metabolic response to food was similar whether the infants were kept at or below the neutral environmental temperature. The crucial importance of temperature in determining the metabolic rate of the newborn baby appears to have been underestimated in many investigations reported prior to 1960. But since the classic studies of Brück (15), it is generally accepted that newborn infants, including premature infants, respond to cold stress with an increase in heat production that can be greater than that due to either activity or food. The metabolic increase in response to cold occurs below a critical temperature which is variable among infants and therefore ideally determined for each experimental situation.

RESULTS

Recent studies of minimal rates of oxygen consumption among low birth weight babies are listed in Table 1. Some of the variation in results may be due to the experimental condition—for example, the high rates reported by Miller et al (17, 18) are probably the result of a sub-thermoneutral environment. There is evidence, however, that heterogeneity of the low birth weight

TABLE 1. Recent Studies of Minimal Rates of Oxygen Consumption Low Birth-Weight Infants

Author	Subjects		Thermal environment	Oxygen consumption ml/kg. min STPD		
	Birth weight range (g)	Postnatal age range		First day	2–10 days	Beyond 2 wks.
Brück (15)	1120–2270	birth–10 weeks	32–34°C air temperature. Radiant and air temperatures similar, babies unclothed.	4.9	5.1	5.3–6.3
Brück, Parmalee & Brück (16)	1220–2210	5–58 days	Same as above.		6.1	7.7 (2–8 wks)
Miller et al (17)	1640–2375	1–54h	31–32°C air temperature.	7.0	7.2	
Miller et al (18)	1680–3025	4h–49h (mean 22h)	31–32°C air temperature, radiant temperature not specified, probably lower.	7.3[a]		
Adams et al (19)	1040–2110	2.5h–18 days	32–34°C, radiant and air temperature similar, baby unclothed.	4.5	6.4	
Mestyán et al (20)	1000–1500 1500–2000	birth–36 days birth–31 days	34.5–36.5°C air temperature, radiant temperature probably similar.	4.3 4.9	4.5 5.1	5.3–6.4 5.7–6.5
Sinclair & Silverman (21)	750–2500	2–10 days	Abdominal skin servocontrolled at 36.0°C.		5.9	
Scopes & Ahmed (22)	<1500 (mean 1276)	1–42 days	Above critical temperature.	4.5	5.3	6.9
	>1500 (mean 1972)	1–42 days	Same as above.	4.5	5.6	7.1
Hill & Robinson (23)	1470±390	birth–14 days	Above critical temperature.	4.9 (12h) 5.7 (12–48h)	5.7–6.3	7.2–8.6
	2190±300	birth–14 days	Same as above.	5.1 (12h) 6.4 (12–48h)	6.4–7.0	8.3–9.0
Hey (24)	1000–1500	4h–22 days	35°C.	5.4	5.8–7.6	7.8
	1500–2000			5.7	6.3–7.5	8.3
	2000–2500			5.3	6.9–8.0	8.5
Krauss & Auld (25)	<1250	birth–21+ days	34.5–36.0°C air.		7.3	8.0
	1251–1500				7.0	7.7
	>1501			6.7	7.6	8.4

[a] Reported by authors at 37°C, converted here to 0°C for ease of comparison.

population is considerable and that gestational differences between babies of similar weight may contribute to the variation in rates of metabolism. On the first day of life, oxygen consumption ranges between 4.3 to 5.4 ml/kg/min. Both Mestyán et al (20) and Hill & Robinson (23) found oxygen consumption on the first day to be slightly higher per unit weight in the large infants among the low birth weight group than in the very smallest. For low birth weight infants as for full size infants, an early postnatal rise in oxygen consumption has been amply confirmed. However, throughout the first ten days of life, oxygen consumption per unit weight tends to be slightly lower for low birth weight than for full size infants of the same postnatal age (15, 21, 22, 24; compare 23 with 26); and lower for those of very low birth weight than for those somewhat larger at birth (20–24). In the late neonatal period (beyond two weeks postnatal age) oxygen consumption per unit weight continues to rise (15, 20) reaching values as high as 8 or 9 ml/kg/min (23, 24, 27, 28). Expressed as calories per 24 hr these figures indicate typical minimal metabolic rates for a low birth weight baby weighing 1500 g at birth as calculated in Table 2.

Small-for-date babies.—Babies who are small for their gestational age may show a particularly marked postnatal rise in metabolic rate, and they systematically tend to have higher rates of oxygen consumption than gestationally less mature babies of similar birth weight. The relative hypermetabolism of the small-for-date infant is suggestive during the first 12 hr (22, 23, 25), but is more easily discernible beyond two or three days postnatal age. Since the original reports of this finding (21, 29), it has been repeatedly noted (22–25, 30, 31). Beyond ten days, metabolic rate in this group continues to be high for weight (22, 23). Sinclair & Silverman (21) proposed that the metabolic requirement of a brain which is disproportionately large in relation to body weight may account for the high rate per unit body weight. In support of this they noted that oxygen consumption of growth-retarded babies, although high for body weight, was not high in relation to head circumference. The marked rise in metabolic rate of fetally malnourished babies over the first days of life suggests that the substitution of the extrauterine for the intrauterine environment has removed a restriction in energy supply that was limiting cellular metabolism. Similar data have been reported in infantile malnutrition where a large rise in oxygen consumption rate has been reported to occur during the early weeks of recovery (32).

Comparison with previous studies.—Hasselbalch (4), in 1904, reported oxygen consumptions of 4.4 and 4.9 ml/kg/min in a five-day-old, 1900 g infant. These values convert to heat productions of approximately 370 and 410 cal/m^2/24h, respectively. Talbot et al (11) were among early investigators who confirmed low rates of heat production among low birth weight infants—for example, 330 and 360 cal/m^2/24h in two infants, aged 3 and 4

TABLE 2. TYPICAL MINIMAL METABOLIC RATES DURING THE NEONATAL PERIOD CONTRASTED WITH THE ADULT.

	Postnatal age	Cal/kg/24h	Cal/m²/24h
Full size baby	birth–6 hr	32	470
3500 g birth weight	latter half of first day	37	550
	second or third day	43	630
Low birth weight baby	first day	34	380
1500 g birth weight	sixth day	42	470
	2–4 wks	50	590
	4–6 wks	59	760
Adult		24	900

Surface area in babies calculated as $A = kw^{2/3}$ using Lissauer's constant ($k = 10.3$).

days, weighing under 1500 g. Later authors emphasized that low birth weight infants have rates of heat production that are similar to full size infants on the basis of body weight, but low when expressed per unit surface area. Thus, Gordon & Levine (33) found an average figure of 58 cal/kg/24th in low birth weight infants in the first weeks of life, a figure practically identical to their experience with full size infants of comparable age, but when they referred the rate of heat production to surface area, the rate was lower in low birth weight infants. Cross et al described similar findings (6).

As pointed out previously, more recent studies have suggested that low birth weight infants have lower rates of heat production than do full size infants, even when expressed per unit body weight. It seems entirely possible that there may have been a systematic underestimation of environmental thermal requirements of the smallest babies in some older studies. Thus, some degree of cold-induced metabolic increase may have been included in the estimates. For example, Gordon & Levine (33), reporting studies of clothed low birth weight infants in 24.6 to 28.5°C air, noted that body temperature was usually falling slightly during the period of observation. Cross et al (6) do not mention either body temperatures or environmental temperatures in their studies.

Reference standard.—In inter-species comparison of adult individuals, the adoption of surface area as a metabolic reference standard has been found to minimize differences. Rubner (34) pointed out a proportionality between surface area and metabolic rate (1000 cal/m²/24h) that held over a wide range of body sizes. Brody developed the argument in further detail (35). It was of considerable interest, then, to discover that newborn infants showed low rates of oxygen consumption for their body surface [e.g., 640 cal/m²/24h (5)], and that low birth weight infants showed still lower rates (less than 400 cal/m²/24h in the first days of life).

Benedict & Talbot (36) considered the "active mass of protoplasmic tissue" to determine the metabolic rate of their subjects. This idea has received increasing recent attention in its application to the immature and growing subject (37, 38). Holliday et al (37) proposed that the high metabolic rate per kilogram body weight of the infant (versus the adult) can be explained by the infant's greater preponderance of metabolically active organs per unit body weight. Sinclair et al (38) explored body compositional differences during the last trimester of fetal growth as a possible determinant of higher heat production per unit weight in larger infants than smaller infants. They suggested that the higher metabolic rates per kilogram of body weight in full size babies were consistent with the known decrease in the relative size of the extracellular fluid compartment during the third trimester of fetal growth.

A second interest attaches to the problem of surface area and body weight as reference standards for the expression of metabolic rates. Table 2 shows changes in metabolic rate with age in a preterm baby weighing 1500 g at birth, expressed either as cal/kg body weight or cal/m^2 surface area. The heat production per unit body weight is lower in the youngest baby, and because the youngest baby has a higher surface/mass ratio, metabolic rate during growth will be seen to increase more than twice as much in relation to body surface as it does in relation to body weight. Thus, the use of surface area as a metabolic reference standard actually augments, rather than diminishes, differences in expression of metabolic rate among neonates. This question is explored in more detail by Sinclair et al (38), and by Hill & Robinson (23).

HEAT LOSS

FETAL

The temperature of the human fetus at term, measured in utero or immediately following delivery, is higher than the maternal reference point (39–42). Mann, in five asphyxiated babies, observed a tendency toward higher fetal-maternal temperature gradient than usual (42). He suggested that terminal impairment of placental perfusion may have contributed to this difference.

No direct measurements have been made in the human to establish the relative importance of the two potential pathways of heat loss from the fetus—the umbilical circulation and the body surface. Adamsons & Towell (40) estimated theoretically that the thermal diffusion capacity of the villous capillaries should considerably exceed that of the body surface. Assuming a thermal diffusion capacity of the placenta at term of 10^5 cal/sec/°C, an umbilical arterial flow of 100 ml/kg/min and a fetal heat production of 25 cal/kg/min, Adamsons (39) predicted a temperature difference between umbilical artery and vein of about 0.3°C if placental flow were countercurrent or 0.6°C if concurrent. In fact, it is neither countercurrent nor concurrent. Cineradioangiography (43) demonstrated that spiral arterioles, opening at the floor of the intervillous space, intermittently discharge "spurts" of maternal blood which enter the intervillous "lake." Into this lake project fetal cotyledons containing complex loops of anastomosing capillaries. This unique double circulation of blood through the placenta does not readily permit mathematical prediction of heat exchange. However, since the fetal-maternal temperature difference is fairly large (about 0.5°C) it does appear that the placental circulation has the capacity for dissipating all, or nearly all, of the heat of fetal metabolism in the human. Some heat might be lost from fetal surface to amniotic fluid, but no experimental observations appear to have been made.

The observation of fetal core temperature in the region of 37.6 to 37.8°C at term raises an interesting but unanswered question about a possible set point change in the neonatal period, as these temperatures are above the sweating threshold of the neonate.

POSTNATAL

In the extrauterine environment, heat generated by metabolism is dissipated along a temperature gradient from the interior of the body to the body surface to the surrounding air. Burton (44) distinguished two portions of the total drop in temperature—the "internal gradient" (rectal–skin) and the "external gradient" (skin–air). Although the baby may exert physiologic control over the internal gradient by regulation of vasomotor activity, Burton (44), Hardy (45), and others have emphasized the purely physical nature of the external gradient. Thermal interchanges with the extrauterine environment occur through radiation, convection, conduction, and evaporation. In a thermal steady-state, heat produced by metabolism is lost by these routes at precisely the same rate as it is produced.

Newborn infants have a relatively large surface area in relation to body mass. The surface-to-mass ratio is particularly large in the infant of low birth weight. However, the surface available for heat loss by radiation and convection is not the total surface but rather a proportion of the total surface which varies with posture. Posture characteristics of various gestational ages, as described by Amiel-Tison (46), tend toward increasing flexion of the extremities with increasing gestational age. Thus, a systematic reduction in the effective surface/total surface ratio would be anticipated in association with advancing maturity. No quantitative description is as yet available of the thermoregulatory benefit derived.

Total basal evaporative water loss averages 10.8 g water/m^2/h in infants 2 to 10 days old at moderate ambient humidity (pH_2O 18 mm Hg) (10). This finding agrees with that of Levine, Kelly & Wilson (47) who, however, expressed their result in terms of body weight (0.9 g/kg/h). A similar estimate was obtained by Day (1), and a somewhat lower estimate by Zweymüller & Preining (48). It has been suggested that in the very smallest preterm infants evaporative water losses may be considerably higher (49).

THERMAL INSULATION

Surface-air insulation.—An estimate for mean ambient insulation of 0.14°C/m²/h/cal for a 3-kg baby may be derived from the data of Hey, Katz and O'Connell (50, 51), a value somewhat lower than that calculated for the adult lying quietly in still air (0.18° C/m²/h/cal). The difference is presumably due to the difference in characteristic dimension (radius of curvature). The lower the radius of curvature of a body, the more easily does it exchange heat with the environment for a given gradient of temperature (45). Thus, a baby would exchange heat more easily than an adult, and a 1-kg baby more easily than a 3-kg baby (51). Typical values for ambient insulation for a newborn baby are given in Table 3.

Tissue insulation.—The insulation provided by the tissues of the baby depends largely on the amount of subcutaneous fat, whose thermal conductivity is low, and on the ability to modify skin blood flow and therefore skin temperature. In situations where blood volume is decreased (e.g., early clamping of umbilical cord) a wider core-skin temperature gradient has been observed (52). As compared with the adult, whose typical value for tissue insulation equals 0.13°C/m²/h/cal, the baby's tissue insulation is low, and varies directly with body weight and fatness. Hey & Katz (50) give a value of 0.05°C/m²/h/cal for a 2.5-kg baby in a cool environment (Table 3). At body weights below 2 kg, tissue insulation decreases substantially. Thus, the ambient and tissue components of total thermal insulation act synergistically to produce particularly low values for total insulation in the smallest infants. This important handicap of small body size is not modified by differences in gestational or postnatal age (51).

Burton (44) noted an apparent paradox between the increase in size of a baby during the first months of life on the one hand, and, on the other, an increase in heat production (and therefore heat loss) per unit surface area over the same period. He deduced that the baby at birth was better insulated than the adult, and lost this advantage with growth, possibly due to a supposed decrease in fat and increase in water content of the tissues, or a progressive increase in the peripheral and general circulation. Body composition studies indicate, however, that water and fat content change in the opposite direction to that required to support Burton's deduction (53, 54). Burton's deduction is demonstrably erroneous, and appears to have been based on the misconception that the environmental temperature at which the metabolic measurements in infants were made was similar to that used for studies on adults.

Conductance, the inverse of insulation, is expressed in units of cal/m²/h/°C of difference between the warm inner core and the temperature of the skin. In a cool environment with skin vessels constricted, a value of 20 cal/m²/h/°C is characteristic for a 2.5-kg baby versus a value of 8 for the adult. The difference is due to the relatively thicker tissues and greater proportion of fat in the adult.

TABLE 3. TOTAL THERMAL INSULATION (°CM^{2}H/CAL). Comparison of baby (2.5 kg, lying on a foam mattress in cool, nearly still air) with adult

	Baby			Adult[c]
Insulation of:	Naked	Clothed[a]	Cot-Nursed[b]	
Body tissues	.05	.05	.05	.13
Air	.14	.14	.14	.18
Clothing	—	.22	.22	.18
Bedding	—	—	.11	—
Total	.19	.41	.52	.49

[a] Dressed in "short woollen vest, large towelling napkin, and long cotton nightdress."

[b] "Flannelette sheet, two layers of cotton blanket."

[c] Business suit.

Adapted from Hey & O'Connell (57).

HEAT LOSSES FROM BABIES IN INCUBATORS

Hey & Mount (55) investigated heat exchange in newborn babies lying naked in a Perspex single-walled incubator of either the natural convection or forced convection type. The main channels of heat exchange were found to be by radiation and by convection. (Heat gain or loss by conduction through the mattress was found to be almost negligible). The mean inner surface Perspex wall temperature was found to be almost half way down the temperature gradient between incubator air and room air. Thus, when the incubator was placed in a cool room the infant suffered a considerable radiant heat loss to the Perspex wall. Radiant heat loss could be reduced by interposing a thin baffle (1 mm thick transparent Perspex which is opaque to skin radiation) between the infant and the incubator wall. The shield quickly reached almost the same temperature as the warm air surrounding it and the radiant loss was substantially reduced.

Raising the relative humidity in incubators decreases evaporative water loss, particularly from the respiratory tract, and consequently lowers heat loss by evaporation (10, 56). Since evaporative heat loss accounts for about one-fourth of the total heat loss in a neutral thermal environment, the 30 percent reduction in evaporative water loss at high humidity noted by Hey & Katz (10) amounts to less than 10 percent of the total heat loss (even less in a sub-thermoneutral environment where radiant and convective losses are relatively greater). It is, therefore, somewhat surprising that there is a demonstrable difference not only in body temperature, but also in mortality, as a function of this small thermal effect (56).

HEAT LOSSES FROM BABIES NURSED IN BASSINETS

The addition of clothing (shirt, diaper, and gown) and bedding (flannelette sheet and cotton blanket) so increases the insulation of the baby that a considerably lower environmental temperature can be tolerated than is the case with the naked baby. Hey & O'Connell (57) confirmed early impressions of Budin (58) and of Blackfan & Yaglou (59) that clothed babies enjoy thermal balance with minimal rates of heat production at an air temperature of 24°C (in the case of the smallest babies, 30°C). Moreover, the provision of clothing minimizes the effect of fluctuation in environmental temperature on heat losses.

PHYSIOLOGIC RESPONSES TO HEAT

VASODILATION

Day (1) demonstrated peripheral vasodilation in premature infants exposed to heat stress. Calculated values for tissue conductance rose from around 20 cal/m^2/h/°C in cool conditions to as high as 80 cal/m^2/h/°C when skin temperature exceeded 36°C. Additional evidence for vasodilation in the warm has since been obtained (15, 50, 50–63).

SWEATING

There was at first some doubt about the ability of newborn infants, particularly low birth weight infants, to sweat. Day (1) detected no consistent sweating in low birth weight babies exposed to rise in environmental temperatures, i.e., many infants did not sweat even though air temperature was raised to 34°C. More recently, Hey & Katz (10) observed that for sweating to be regularly elicited in newborn babies, environmental temperatures higher than Day's must be used. They found a threshold of approximately 37.2°C rectal temperature for increase in total evaporative water loss, with a tendency for the threshold for sweating to fall with postnatal age. They showed that term infants have the capacity to treble evaporative water loss; but in infants born more than three weeks before term, a smaller response occurred, and sometimes none at all. A similar response was seen in normally grown and small-for-date babies of the same gestational age. Visible sweating appeared first on the forehead and temples, later on the chest, and usually by 242–260 days post-conceptional age, on the legs. No notable changes occurred in respiratory frequency, indicating that a respiratory contribution to the increase in total evaporative water loss is probably small.

The density of active sweat glands on the thigh of the baby at term is more than six times that of the adult. However, the mean peak sweat rate following intradermal acetylcholine stimulation (64) is only one-third of the maximum rate recorded in adults. Although the sweat response to thermal stimulation in the first ten days after birth is lower than in later life, it cannot be governed by glandular limitations in the term infant. In the preterm baby, however, the inability to sweat in response to either thermal or exogenous acetylcholine stimulation may be a function of incomplete development and differentiation of the sweat glands (64).

PHYSIOLOGIC RESPONSES TO COLD

PERIPHERAL VASOCONSTRICTION

Brück (15) showed that constriction of skin blood vessels occurred in response to body cooling in both full size and low birth weight infants. During cooling, vasoconstriction in the heel was the first thermoregulatory change recorded, preceding any change in oxygen consumption. Maximal constriction was reached when mean skin temperature was still above 35°C.

The effect of peripheral vasoconstriction in response to cold is to increase the internal (core-skin) gradient of temperature, and to increase tissue insulation, and therefore total insulation, to their maximal values. But, despite the fact that the newborn's range of control over tissue insulation is similar to that of the adult (50), his maximal tissue insulation is low by comparison. This defect is essentially a function of small body size.

INCREASE IN HEAT PRODUCTION

Mordhorst (65) and Day (1) first demonstrated that environmental temperature influences the rate of heat production of babies; and the studies summarized by Brück (15) definitely established the relationship.

Brück et al (15, 60, 61) made the following observations regarding the relationship between environmental temperature and metabolism in newborn babies. When environmental temperature is lowered from a neutral point (32–34°C in the metabolic chamber used by Brück), the infant's rate of heat production rises. Both low birth weight and full size babies show this response, although the metabolic rise is greater among full size babies in the first ten days of life. In both groups of babies, the cold-induced metabolic rise is present in the first hours after birth, but is less on the first day than thereafter. The cold-induced increment, measured acutely, is roughly proportional to the cold stress applied—i.e., the response to 23°C exceeds the response to 28°C air. The maximal response is about 2.5 times the resting rate of heat production. The metabolic rise in response to environmental cooling may occur before there is any fall in rectal temperature; conversely, during warming of a cool infant, the rate of heat production starts to fall from elevated to minimal levels before any rise in rectal temperature occurs. Thus, during warming or cooling, there is no relationship between rectal temperature and rate of heat production. The acute response to cold is mediated via cutaneous thermal receptors and is seen when the average skin temperature is lowered to about 35–36°C. The metabolic increase is not always successful in preventing a fall in rectal temperature, particularly in low birth weight infants and in all babies in the first day of life. Infants in a cold environment are restless, often cry but do not appear to shiver unless there is a rapid drop in rectal temperature. There is not a clear relationship between the level of heat production and the amount of muscular activity in the cold; thus, chemical thermogenesis may contribute.

Subsequent investigators have, in general, amply confirmed the validity of Brück's observations, and have developed some of the foregoing points in more detail (24, 26, 66–69).

The metabolic response to cold dwarfs the metabolic increase due to specific dynamic action or even to activity. The increase in metabolic rate with fall in environmental temperature is approximately linear; the response can be described as a slope (change in oxygen consumption per kilogram/change in environmental temperature), which can be examined for babies of differing size and age. For low birth weight babies the response appears to be quantitatively smaller than for full size babies on day one (15, 24). However, the response of the low birth weight infant rises with increasing postnatal age, so that the baby who is more than three weeks old typically shows the most impressive response of all. It is important to note that these represent acute responses; there is no guarantee that they can be maintained (69). Moreover, these reactions can be modified by illness (15, 70, 71) or by the thermal environment in which the baby is raised (72).

Relation of metabolic rate to body temperatures.—Hey & Katz (73) reported minimal rates of oxygen consumption in newborn babies when the abdominal skin temperature was near 36.2°C (full size babies) and 36.5°C (low birth weight babies). Silverman et al (69) examined the effect on oxygen consumption of servo-controlling the exposed abdominal skin of infants of very low birth weight (less than 1500 g) at 35, 36, 37°C. Mean oxygen consumption was lowest in the 36°C condition, and rose 11 percent on the average, when abdominal skin was controlled at 35°C.

Brück (15), Silverman et al (69), and Hey (24) observed a fall in rectal temperature during cold exposure in low birth weight babies making appropriate homeothermic responses. Although the fall is typically largest in the least mature babies (68), it occurs also in term babies. The failure to achieve a completely constant rectal temperature despite the appropriate qualitative homeothermic responses has been consistently observed on occasions when heat production was well below the maximum that could be achieved. Some observers (16, 69, 74) have suggested from these findings that the body core temperature is regulated at a lower level in the newborn exposed to cold.

Mechanism of cold-induced metabolic rise.—Although an increase in muscular activity, with or without shivering, undoubtedly contributes to the increased heat production of the low birth weight infant exposed to cold (30, 74), several observations have suggested that it does not account for the whole of the metabolic rise. Day (1) found that changes in visible muscular activity could not be fully correlated with variations in oxygen uptake, an observation confirmed by Hill & Rahimtulla (26), Oliver &

Karlberg (75), and Hey (24). Hey (24) studied the metabolic response to cold in seven infants before and after sedation with chloral hydrate (60 to 80 mg/kg body weight). When sedated, they remained quiet and apparently asleep at low environmental temperature, and no muscular activity could be seen (or detected electromyographically in three infants). Oxygen consumption increased significantly at the lower environmental temperatures, but the response was only about half of what it was in the unsedated infant. Some increase in general muscle tone may have occurred, but the suggestion is strong that nonmuscular thermogenesis occurs in the newborn baby in response to cold.

Major attention has recently been directed to the role of cold and catecholamines in stimulating metabolism in brown adipose tissue. Aherne & Hull (76) have described the presence of brown adipose tissue in newborn human infants in five sites: a mass lying in a thin diamond-shaped sheath between the scapulae, smaller masses around the muscles and blood vessels of the neck (internal jugular vein and common carotid artery), large deposits in the axillae, moderately large masses in the mediastinum between the esophagus and the trachea as well as deposits around the internal mammary vessels, and a large mass around the kidneys and adrenals.

In the human, primitive brown fat cells begin to differentiate from reticular cells at about 26 to 30 weeks. Development of brown adipose cells is not complete at term, for by the third to fifth postnatal week the amount of cytoplasm per cell has increased to over 150 percent of that seen at birth (76).

There is indirect evidence that brown fat plays a role in the response of human babies to cold. Karlberg, Moore & Oliver (77) showed a rise in oxygen consumption in human infants following norepinephrine infusion. In some infants, the metabolic response was accompanied by arousal and increased physical activity; in others it occurred without appreciable change in activity. Stern and co-workers (78, 79) observed concomitant increases in oxygen consumption and urinary catecholamine excretion (norepinephrine and dopamine) in newborn infants subjected to a cold stress. Restlessness, but no visible shivering, also occurred. A significant increase in plasma nonesterified fatty acid levels was also seen (78). Norepinephrine infusion produced a similar rise in plasma nonesterified fatty acids. Stern (80) and Lees, Younger & Babson (30) have found that some very small premature infants do not increase either urinary norepinephrine excretion or rate of oxygen consumption when challenged by cold, and show a large fall in body temperature in the face of this deficient response.

Silverman et al (81) measured surface temperatures in a group of neonates exposed to moderately cool conditions for an observation period of 1 to 4 hr. The exposed surface temperatures of the extremities and trunk fell sharply while the temperature of the nape fell significantly less than other surface areas. On three occasions the temperature of the nape was actually higher after one hour of cooling than at the onset. Similar findings have been reported by Grausz (82). These findings suggest but do not prove that increased metabolic activity and perfusion of nuchal brown fat occurs in human infants during cold exposure. Dawkins & Scopes (83) noted that the elevated oxygen consumption of newborn infants during mild cooling is associated with increased plasma glycerol levels but no change in plasma-free fatty acids. Since triglyceride lipolysis in fat slices results in less free fatty acids being released from brown fat than from white fat (84), these data were taken to represent brown fat thermogenesis. Heim et al (85) compared histologically the brown adipose tissue from babies who died after having been nursed either in incubators (34–35°C) or who were conventionally swaddled and nursed at room temperature (23–27°C). The tissues studied from the infants nursed in warmer conditions were replete with fat but 83 percent of the brown fat derived from infants conventionally swaddled exhibited fat depletion.

Studies to date indicate that norepinephrine stimulates brown fat metabolism by stimulating production of cyclic 3, 5-AMP which, in turn, activates a lipase catalyzing triglyceride hydrolysis. Both the released fatty acids and glycerol can be readily oxidized in vitro by the brown fat, and it appears that either or both may serve as the fuel during norepinephrine stimulation of brown fat metabolism. The biochemical mechanism by which norepinephrine can induce a sustained elevation of the rate of brown fat oxygen consumption is not yet completely understood. It has been proposed that norepinephrine indirectly uncouples oxidative phosphorylation in brown fat mitochondria by elevating the intracellular level of fatty acids (see review by Smith & Horwitz, 86).

A baby's need for oxygen enrichment of the inspired air theoretically can be tested by measuring his ability to produce heat by nonshivering thermogenesis. Oliver & Karlberg (75) and Scopes & Ahmed (71) showed that moderate hypoxia impairs the ability of the newborn baby to increase oxygen consumption in response to cold exposure. In premature babies with hyaline membrane disease, this response was impaired as arterial PO_2 fell below 45–50 mm of mercury, and abolished as PO_2 fell below 30 mm of mercury. A fall in rectal temperature resulted when the cold stress was not compensated by increased heat production. Raising the inspired oxygen concentration often restored a normal metabolic response to cooling and caused rectal temperature to rise, suggesting that the baby was hypoxic previously, and that adding oxygen to the environment had corrected the hypoxia (71).

REGULATION OF BODY TEMPERATURE

THE SET POINT

Central.—At rest in a neutral thermal environment, adult man has a rectal temperature of 36.5–37.5°C. But in low birth weight infants, particularly those under 1500 g, Day (1), Silverman et al (69), Mestyân et al (20), and Brück (15) have observed rectal or colonic temperatures that are sometimes lower (less than 36.5°C) than generally considered normal in humans, despite other evidences of thermal comfort—e.g., minimal oxygen consumption rate, quiet sleep (16). This finding has suggested to some investigators that the "set point" of the deep body temperature is below the range of 36.5 to 37.5°C in some young and small infants. A similar downward set point deviation has been described in cold-acclimated adult humans (87).

The precise mechanism by which resetting of the thermostat may be accomplished has not been determined. Recent observations in guinea pigs suggest that shift of the thermoregulatory set point originates from a change in the activity of norepinephrine-sensitive, thermo-insensitive neurons, serving as "reference signal units" for the thermoregulatory system, located in the anterior hypothalamus but distinct from the preoptic thermosensitive units (88).

Peripheral.—Despite the fact that adult man shows skin temperatures of 33 to 35°C when at rest in a thermoneutral environment, babies show a brisk metabolic rise when skin temperature falls to this range (15, 69). It appears, from the observations of Brück, that a thermoregulatory drive originates in the periphery since, on exposure to cold, both vasoconstriction and nonshivering thermogenesis can occur without any change in core temperature. Mestyân et al, moreover, showed not only that surface temperature is important in evoking the metabolic response to cold (89), but also that the face is particularly sensitive to thermal stimulation since the local application of a cold stimulus to the face resulted in a distinct metabolic rise (90). Brück (15) emphasized the newborn infant's greater sensitivity in regard to cutaneously induced metabolic rise as compared with the adult, and speculated that this difference could be due either to a threshold difference in the cutaneous thermal receptors, or a greater response to the same influx of efferent impulses. In this connection, it is important to recall the newborn infant's comparatively low value for heat loss per unit surface area in a thermoneutral environment. A small increase in the skin-environmental temperature gradient may comprise a substantial threat to thermal balance.

CONSEQUENCES OF THERMAL STRESS

Evidence has accumulated on the effects of prolonged cold stress on the survival, growth, and maturation of the low birth weight infant.

Survival.—Silverman et al (91), Buetow & Klein (92), and Day et al (93) all observed an increased mortality in the first days of life in low birth weight infants raised in incubators providing a thermal environment below the neutral range. Silverman & Blanc (56) observed a lower survival rate in low birth weight infants reared in incubators providing moderate humidity (30 to 60 percent relative humidity) rather than high humidity (80 to 90 percent). But it was suggested that this decreased survival was due to a thermal effect—i.e., heat loss by evaporation was greater in the less humid environment; and it was subsequently determined that there was no significant nonthermal advantage to "high" over "moderate" relative humidity (94).

The mechanism of the increased mortality in low birth weight infants exposed to environments below the thermoneutral range has not been determined. No distinctive or unique cause of death was revealed by postmortem examination of infants included in the controlled trials enumerated above. The trials were performed before much of the presently available information about the newborn's capacity for nonshivering thermogenesis had been acquired. Consequently, some critical clinical and pathological observations were not made (e.g., metabolic rate, oxygenation, lipid content of brown fat at necropsy). It is attractive to speculate that the energy cost of defending body temperature against cold contributes to the lethal effect, particularly in the first days of life, when the balance between caloric supply and caloric expenditure is marginal, and particularly in low birth weight infants whose glycogen and fat stores are limited. However, it is also possible that lower body temperature per se is a contributing cause. Studies utilizing animal models may help decide which of these two possibilities is correct.

Cold resistance.—Hahn and co-workers (95) found that newborn rats raised at 3°C and 22°C were better able to prevent a fall in rectal temperature during acute cold exposure than were controls raised at 33°C. The cold-exposed rats showed differences in the development of the thyroid gland (greater follicle size and epithelial height) which suggested greater functional activity. Brück & Wünnenberg (96) found evidence in newborn guinea pigs that nonshivering thermogenesis is almost completely replaced by shivering within four weeks in animals reared at the neutral temperature (30–32°C). Conversely, the replacement of nonshivering by shivering thermogenesis could be retarded but not completely inhibited by rearing the guinea pig at 8°C. These observations suggest the concept that the reduced capacity for nonshivering thermogenesis that accompanies postnatal development in the guinea pig is a developmental process that can be modified by environmental temperature.

In the human baby, however, there is evidence that brown fat continues to develop for some weeks after birth (76) as does capacity for nonshivering thermogenesis, particularly in premature infants. Glass et al (72) tested the effect of environmental temperature on this developmental process. They found that low birth weight infants, raised between one and three weeks postnatal age in slightly sub-thermoneutral conditions, had improved cold resistance at the end of that interval, whereas matched controls, fed isocalorically, did not. The improved cold resistance was associated with an increased capacity to increase oxygen consumption in response to a cold stress.

Growth.—Glass et al (72) also explored rates of growth in low birth weight infants continuously exposed to contrasting thermal environments between one week and three weeks after birth. Although each group received identical feeding (120 cal/kg/24h), the babies raised in the sub-thermoneutral condition grew more slowly because they increased their rate of heat production to maintain body temperature. They were able to make up this difference when given a caloric supplement sufficient to match the calculated cost of the cold-induced metabolic rise (97).

These recent demonstrations of the effect of the thermal environment on growth recall Blackfan & Yaglou's (59) earlier demonstration that air temperature and humidity affect postnatal weight loss and growth of infants of low birth weight.

Neonatal Cold Injury

Neonatal cold injury, a clinical syndrome first clearly described by Mann (98) and Mann & Elliott (99), consists of very low body temperature, slowness or refusal to feed, edema, lethargy, redness of the face, and, often, localized hardening (sclerema) of the subcutaneous tissue. The blood sugar is often low, and the blood urea nitrogen is elevated. The syndrome apparently results from prolonged exposure to a cold environment, and chilling immediately after birth appears to contribute in the majority of cases. Those who die usually show massive pulmonary hemorrhage, and coexistent infection is not uncommon.

Progressive lipid depletion of brown adipose tissue is noted over the first few days of life in human babies who come to autopsy (76), particularly in those who have been exposed to cold during life (85). It is unusual to find brown adipose tissue wholly depleted of lipid at necropsy, but Aherne & Hull (76) collected a small series of such cases. Prominently represented among this group were babies who were small-for-date and who suffered total depletion of their brown adipose tissue within 3 or 4 days of birth; and, secondly, infants who were admitted to hospital with neonatal cold injury, and died with massive pulmonary hemorrhage, and with brown fat wholly depleted of lipid at a mean age of 8 days. Newborn rabbits whose adipose tissue is depleted of lipid, and who cannot produce extra heat in response to cold (100), recover the ability to do so after milk feedings (101). Thus, it seems probable that neonatal cold injury results from a vicious circle, involving cold exposure, fall in body temperature, heat production in brown adipose tissue, lipid depletion, failure of metabolic response, further fall in body temperature, lethargy and loss of appetite, and failure to replenish lipid stores. Hey & Katz (70) documented temporary loss of the metabolic response to cold in three infants of low birth weight. They also noted that these infants were in both sub-thermoneutral environments and negative caloric balance throughout the first week of life, and suggested that exhaustion of fat stores available to brown adipose tissue could have been responsible for the temporary loss of response to cold stress. Thus, a "forme fruste" of neonatal cold injury may occur not uncommonly in low birth weight babies in the first week or two of life.

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NUTRITION, ENVIRONMENT, AND BEHAVIORAL DEVELOPMENT[1]

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INTRODUCTION

During the past 15 years, a number of studies involving several different scientific disciplines have associated malnutrition imposed during a critical period of development and permanent changes in brain structure and function. The implications of the observations in a world in which hundreds of millions of children have been and are continuing to be nutritionally deprived in infancy are obvious. Not only are they to be handicapped, but the resulting handicap may well prevent them from extricating themselves from the conditions which breed malnutrition, and therefore their children are at risk of suffering the same handicap. Thus, poverty results in undernutrition which causes retarded brain growth and function which limits earning capabilities which lead again to undernutrition. This is the vicious cycle first pointed out by Cravioto (1).

This association of undernutrition and faulty development has been made in complex human situations and thus has not allowed isolation of malnutrition as the only significant variable. Malnutrition occurs in a milieu of low socioeconomic status, reduced education, poor sanitary conditions, and is further complicated by recurrent and severe infections. These children, then, are exposed during their early formative life to a variety of complex environmental stimuli only one of which is undernutrition.

In an attempt to isolate malnutrition and to explore the interrelations between malnutrition and other factors in the poverty environment, a number of epidemiological studies have been attempted in humans, and animal models have been sought.

ANIMAL STUDIES

Animal models provide several advantages when compared to human field studies. The diets can be closely controlled and frequently analyzed to ensure uniform composition. Furthermore, the extreme and extended effects of severe malnutrition can be studied, the study of these effects being excluded from human studies for obvious ethical reasons. The laboratory also provides a setting in which environmental and social conditions can be made uniform and constant during both the period of malnutrition and subsequent recovery.

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MALNUTRITION AND LEARNING

The initial efforts of investigators of behavior and malnutrition were directed toward the effects of malnutrition on subsequent learning ability either during the period of malnutrition or after refeeding (2–5). These studies purported to demonstrate poorer learning, using the Hebb-Williams maze over one trial on each problem after a period of familiarizing the subject with the environs of the testing chamber. Poorer performance in the malnourished subjects was found during both the period of malnutrition and after recovery, the latter period showing results less significant than the former but none the less apparently permanent in nature. A critical review of these studies by Levitsky & Barnes (6) pointed out that the use of the one trial for each problem is not a valid test of learning in the Hebb-Williams maze, but rather reflects only the performance of the subject in accomplishing the particular task put before it. Furthermore, these authors again point out that the use of food reward (in the above cases a mixture of the low protein and control diets was utilized) has different incentive values for malnourished, previously malnourished, and control animals (7, 8). Consequently, reported differences in the learning process per se may, in fact, not represent learning but rather reflect performance in a learning paradigm which is influenced by differences in motivation, emotionality, and incentive value of food reward between malnourished and control animals. A subsequent study (9), using the Hebb-Williams maze over eight trials on each problem but retaining the food reward, were felt to confirm the authors' earlier reports of differences in learning though again the influence of food reward makes interpretation difficult.

Barnes et al (10) demonstrated the poor ability of adult, previously malnourished rats compared to well-nourished controls to perform in a test of visual discrimination in a water maze. This study demonstrated that in a "nonstressful" situation (water temperature 18°C) the malnourished rats did not attain the same quantitative level of performance as the controls though both groups were able to solve the problem in the same qualitative manner. Cowley & Griesel (11) had previously shown that in the water Hebb-Williams maze performance of the previously malnourished rats was reduced in a "nonstressful" situation, and, further, that the difference widened under a more stressful situation (water temperature 10°C). In the light of these two studies (10, 11), and a third study (12) showing that reactions of previously malnourished animals were different toward adverse stimuli (reflected by increased bar pressing rates to electric shock or for food reward), it is difficult to interpret whether the results reflect differences in learning per se or are totally confounded by the proven differences in motivation and emotionality.

In monkeys, a recent report (13) indicates that the qualitative acquisition of problem solving on either easy or difficult puzzles is the same between subjects malnourished after weaning and well-fed controls; the final level of performance, however, is significantly reduced in the malnourished monkeys. Another study in nonhuman primates (14) substantiates these findings though the use of food reward again opens this study to criticism. Further studies in rats (15–17) and pigs (18) in learning paradigms report differences in performance using electric shock as reinforcement. In these studies it is interesting to note that the authors report increased "nondirected" activity in the previously malnourished animals (thought to reflect heightened levels of emotionality) during testing.

In the light of past studies and the criticisms mentioned above, it is impossible to conclude at this time that malnutrition negatively affects the learning process per se, but it apparently does bring behavioral responses which compromise the ability of an animal to perform in a classical learning paradigm.

Further Indication of the Effects of Early Malnutrition on Emotionality

Measurement of the effects of malnutrition on emotionality have most commonly been carried out in the open field, measuring both horizontal (number and position of squares traversed) and vertical (rearing and head lifting) activity as well as fecal and urinary excretion upon exposure to the novel testing chamber. This paradigm offers certain advantages over those discussed with regard to learning in that no reward or other form of reinforcement is required in observing the ongoing behavior of the test subject. Results show that rats protein-malnourished during gestation (19), lactation (20, 21), lactation and a period of postweaning (22–26), or only in the postweaning period (27) show significantly greater emotionality in the open field as demonstrated by decreased locomotor activity (horizontal movement), decreased rearing, head raising and pivoting (vertical activity), increased excretion of urine and feces and decreased time spent in the central squares of the field. These results may also be interpreted as showing reduced exploratory activity contingent on measurements of total activity in previously malnourished rats. Furthermore, all of these observations are intensified to a greater degree in the previously malnourished subjects than in well-fed controls following a loud noise introduced during testing (28, 29). Other investigators (30) report similar increases in emotionality of pigs during testing in a modified free environment after either restricted or low protein intake during the first eleven weeks of life, compared with testing after refeeding. Similarly, in nonhuman primates (31), heightened emotionality has been reported following introduction of the test subject to a novel object (coined a neophobic response).

Summarizing then, the evidence seems quite sound that in different species subjected to a period of poor nutrition during a variety of periods of development, there is a change in the response of the animal to novel situations which is interpreted as reflecting increased levels of emotionality when compared to well-fed controls.

OTHER BEHAVIORS AND MALNUTRITION

The development of certain reflexes (startle, grasp, visual placing, as examples) as well as certain physical parameters (eye opening, incisor eruption) has been shown to be delayed in malnourished animals (32, 33). These delays cannot help also delaying the development of certain behaviors dependent on the maturation of the above-mentioned parameters. In addition, previously malnourished animals show heightened sexual behavior, and greater hunger and hoarding of food, when compared to well-fed controls (34). These observations add further to the evidence that the array of behavioral responses available to the malnourished animal is different from that of its well-nourished counterpart

EARLY ENVIRONMENT BEHAVIORAL DEVELOPMENT

During the past ten years, a body of information has appeared which indicates that environmental parameters in early life have a profound effect in determining qualitatively and quantitatively the final complement of behavioral responses available to an animal as an adult. The most commonly used forms of laboratory stimulation have been handling, electric shock, and exposure to cold during the immediate postnatal period up to 21 days of life. Levine & Denenberg (35) have described the behavioral effects of such treatments as indicating decreased emotionality increased exploratory behavior and decreased reactions to stress (or otherwise adverse stimulation) in the handled animals when compared to their nonhandled controls. In all cases, the subjects were maintained under identical good nutritional conditions. In addition, rats handled in early life show increased body weight and increased survival time when subjected to total food and fluid deprivation as adults (36). Differences in adrenocortical activity are demonstrable in handled versus nonhandled rats, the rat pups during handling showing increased levels of corticosterone in the plasma (37–40), and later as adults, after an adverse stimulus, quantitatively showing reduced responses when compared to their previously nonhandled counterparts (38, 40). Indications are that the degree of stimulation in early life is well correlated with later emotionality in the form of a U-shaped function (41); that is, there exists an optimal level of stimulation above which the degree of emotionality again shows an increase.

From these data, then, it is apparent that early experience, its nature and degree, plays an important part in determining the later behavioral response, adrenocortical activity, and perhaps growth of the animal.

MALNUTRITION AND ENVIRONMENT: SOME POSSIBLE CORRELATES

Several behavioral, somatometric, and biochemical similarities appear to exist between the effects of early malnutrition and early environmental deprivation. Animals exposed to early environmental stimulation display reduced emotionality (42, 43), greater body weight (44), greater brain weight (45, 46), earlier myelination (47), greater dendritic branching (48), increased rate of formation of new cells and suggestion of an increased number of cells in the brain (49), and increased levels of acetylcholinesterase (50, 51) when compared to nonstimulated controls. Isolated rats, compared to their social controls, show increased emotionality (52) and increased levels of acetylcholinesterase expressed as activity/gram wet weight/hour. Expressing these results as activity cholinesterase/activity acetylcholinesterase, and thus independent of brain weight, the brains of isolated animals show reduced levels of acetylcholinesterase activity compared to their social control counterparts (53). Early malnutrition produces similar changes in the isolated and opposite changes in the stimulated animal with respect to increased emotionality (54–56), decreased body weight and cell number (57), decreased brain weight (57, 58), decreased myelination (58–61) and reduced dendritic arborization (62), and decreased levels of acetylcholinesterase during malnutrition (63, 64), changing to increased levels/gram wet tissue in refed adults (65, 66).

These results strongly suggest not only the obvious need for strict environmental control during experiments involving malnutrition, but also that early environment and malnutrition may influence similar tracts of development during the early postnatal period.

INTERACTION BETWEEN EARLY ENVIRONMENT AND EARLY MALNUTRITION

One approach to producing early malnutrition in the rat has been to increase litter size during lactation. By increasing litter size, however, one not only reduces the amount of milk for each pup but also increases the amount of sibling stimulation as well as decreases the amount of attention given by the dam to each individual pup (67). Exploring these latter variables to a greater degree, Frankova (68) reported that there was a considerable behavioral difference between litters of nine and thirteen when compared to a litter size of four or seventeen. More specifically, though there existed an inverse relationship between body weight and litter size (the smallest body weights found in the largest litters), measurements of exploratory behavior and spontaneous activity in the open field proved to be greatest in intermediate litter sizes ($n=9$ and 13) with the smallest ($n=4$) and largest ($n=17$) litters following in that order. She concluded that there existed an interaction between the amount of sibling stimulation (environment) and neonatal nutrition in the determination of exploratory behavior and emotionality in the adult animal. Further substantiation of this effect has been recently made by Levitsky & Barnes (69), using a 2×3 factorial design in which animals either well-nourished or protein-malnourished for seven weeks were subjected to environmental stimulation (handling and a daily period of environmental complexity postweaning), isolation or normal social conditions. Behavioral testing after refeeding revealed a highly significant interaction between isolation and early malnutrition with regard to locomotor activity and exploratory behavior, the isolated malnourished group showing much greater signs of emotionality than either the well-nourished or the other malnourished groups. Furthermore, the same authors showed that environmental stimulation when combined with early malnutrition has redeeming effects, the stimulated-malnourished group falling very close to both the stimulated and nonstimulated well-nourished animals in locomotor and exploratory behaviors. This, indeed, may be the first clear-cut proof that environment and nutritive state effectively combine and interact in the determination of behavioral competence in the adult animals.

POSSIBLE MECHANISMS OF INTERACTION BETWEEN EARLY ENVIRONMENT AND MALNUTRITION

The preceding summary of data currently available leads investigators to speculate as to the nature of the interaction which seems to be evolving between environment and malnutrition in the first few weeks of life, and how this might influence the final behavioral state of the adult animal. Levitsky & Barnes (70) suggest that malnutrition may produce a condition in which the animal is less susceptible to environmental programming and hence, in that experience is cumulative, may render the malnourished animal substantially retarded in behavioral development. Isolation further provokes this condition by additional decreases in the amount of information available to the developing animal. Hence, with fewer earlier experiences, the animal is limited as an adult as to the variety and magnitude of environmental stimuli with which it is able to cope. Furthermore, looking at the effects of malnutrition on a purely behavioral basis, the behaviors elicited in malnourished animals may be incompatible with the gathering of environmental input necessary for the normal development of the processes of socialization and mental development.

Perhaps most encouraging is the fact that preliminary evidence suggests that enriched environment does have possible redeeming effects in the malnourished animal. This possibility, however, will require a great deal more consideration and study before the final answer is clear.

HUMAN STUDIES

Neurologic abnormalities, such as apathy and lethargy, are prominent symptoms of severe malnutrition in children. Both specific and nonspecific electroencephalographic changes also may occur. However, all of these changes are variable and usually disappear with rehabilitation. Gross neurologic sequelae are rare. However, subtle neurologic changes have not been looked for systematically. It would seem to us that this should be a fruitful area for future clinical investigations.

The behavior of children who were severely malnourished as infants has been studied both retrospectively (71–74) and prospectively (75–78). It is difficult, however, in both these types of studies to isolate malnutrition as the cause of the mental deficiencies found. These children invariably come from poorer backgrounds, lower socioeconomic conditions, and a generally more deprived atmosphere than even the most carefully matched control groups. Both types of studies also suffer from the lack of standardization of intellectual tests. Tests developed in advanced countries may have little meaning in developing countries, especially if large cultural differences exist. In addition, retrospective studies, although able to examine more children, suffer from not being able to be sure of the criteria used to establish the diagnosis of malnutrition. However, even with all these problems both types of studies provide valuable data, especially if the data are not overinterpreted and especially when they are considered in the light of animal experiments and physical and chemical studies.

In a retrospective analysis, Cabak & Najdanvic (79) demonstrated that Serbian children with a history of marasmus had significantly lower intelligence quotients than Serbian children in general. This study made no real attempt to control other environmental factors but used controls of the same racial or genetic stock. One important aspect of this particular study is that it deals with malnutrition during the first year of life. Precise time distinctions are often not made in other studies investigating malnutrition in childhood. It is interesting that this study demonstrates one of the largest intelligence quotient deficits of any of the many investigations, and that the patients studied were the youngest when nutritionally deprived. The major weakness in this investigation is the lack of an adequate control group. Normal Serbian children have a certain potential not reached by the study population, but the cause of the retardation cannot be ascribed specifically to malnutrition. The control children not only were better nourished but came from much better socioeconomic environments.

Other retrospective studies in developing countries throughout the world in which more careful control groups were chosen, suggested again that early malnutrition interfered with subsequent learning ability. More recently, Chase (80) has reported that infants in our own country who were severely malnourished early in life performed consistently poorly when subsequently tested at a later age.

A number of prospective studies have been done or are currently being done. Stoch & Smythe (81), studying South African children, have shown that those malnourished early in life are smaller than a control population and have reduced head circumferences and intelligence quotients even after long-term follow-up. Intelligence quotient testing has been adapted for South African children and would appear to be valid. However, again, the control population leaves much to be desired. The malnourished children lived in inadequate housing with no sanitary facilities, came from broken, poverty-stricken homes, and were almost entirely neglected. Control families lived in neat brick houses which had sanitary facilities; all the fathers and mothers were employed and all the children attended nursery school. Again, we see retardation but cannot isolate nutrition as the cause. However, the study dramatically points out the long-term effects produced by this complex of social ills, of which malnutrition is certainly a prominent member.

This problem of control populations is not easily solved by using control groups from "matched socioeconomic backgrounds." Garrow & Pike (82) have attempted to employ as a control group siblings not having a history of hospitalization for malnutrition. Their data indicate that the malnourished group does catch up to the control group. Here we see the opposite problem. Under these conditions, the control children probably were also malnourished, certainly subclinically, and perhaps even clinically. Thus, the authors may be comparing malnourished children with malnourished control children. It is significant that both groups of these Jamaican children had poorer growth than generally accepted Jamaican norms, and significantly retarded growth by United States standards. The problem becomes exceedingly difficult since one must study similar populations in which nutrition is the only variable.

One of the best series of studies to date is that of Cravioto and others (83–85) in Mexico and Guatemala. They have studied populations of uniform socioeconomic backgrounds. In these investigations, performance on psychological tests was found to be related to dietary practice and not to differences in personal hygiene, housing, cash income, crop income, proportion of income spent on food, parental education, or other social and economic indicators. Moreover, these investigators found that the performance of both preschool and school children on the Terman-Merrill, Gesell, and Goodenough draw-a-man tests was positively correlated with body weight and heights. These tests had been previously adapted for the Mexican population being studied. Further investigations in collaboration with the Institute of Nutrition of Central America and Panama (INCAP) in Guatemala again showed a positive correlation between size and performance. The tests included placing blocks in openings, tracing block shapes, and differentiating block shapes by feel alone. These tests were considered to be measures of visual, haptic, and kinesthetic sensory integration, respectively. This study has been interpreted as demonstrating perceptual defects in children exposed to severe early malnutrition. Again, great care was taken in these studies to standardize testing procedures and to eliminate variables other than malnutrition. Although not completely successful, these studies are of the general type that should supply more precise data.

It is significant that in these studies the earlier the malnutrition, the more profound is the psychological retardation. The most severe retardation occurred in children admitted to the hospital under 6 months of age. Children admitted later with the same socioeconomic background and the same severe malnutrition but a different time of onset did recover with prolonged rehabilitation. This recovery of older children, even when severely malnourished, has been observed before. Kugelmass and associates (86) demonstrated that rehabilitation in a group of malnourished New York children significantly increased their intelligence quotient scores. Thus, it is possible that time rather than nutritional state may be of paramount importance in determining whether or not sequelae will be permanent. Data from a study (87) still not complete indicate that Chilean children admitted to a special nutrition unit with severe marasmus below 6 months of age remain retarded in physical, motor, and mental development at least until 5 years of age (when the last testing was done).

It is interesting that these survivors of marasmus are from the same population and were admitted to the same unit as those who succumbed and showed marked physicochemical changes. Thus, if the child died he showed reduced brain cell number and myelin content. If he survived he was permanently stunted in size and retarded in development. This is the first study, with all its obvious drawbacks, which attempts to correlate changes in brain structure with those of brain function.

The studies outlined above have attempted to "isolate" malnutrition as the significant variable, but have not dealt with the problem of the interaction of early environmental experience with nutritional status. As seen in the previous section, animal experiments strongly suggest that environmental deprivation and malnutrition may act synergistically on development of the central nervous system.

One study has attempted to deal with this possible environment-malnutrition interaction using human subjects. Yatkin & McLaren (88) studied the effect of enriched environment versus social control during rehabilitation in marasmic children. Both groups of children were on identical diets. They showed significantly greater improvement in the enriched group on the basis of developmental quotients (DQ). However, two factors leave the work subject to criticism. DQ testing procedures which showed the greatest improvement in the enriched group were very close to the same procedures used as the enrichment stimulus, i.e., personal-social, hearing, and speech. Secondly, the lack of well-nourished controls subjected to the enriched or social control environments make premature the interpretation that the observed phenomenon is an example of a malnutrition–environment interaction. Indeed, by including well-fed controls one might gain further insight into the first criticism as well. There are two other studies just beginning which are also attempting to deal with this problem, but they are too recent to have shown any results. In this regard, Warren (89) has suggested using another type of control. In addition to matching children for chronological age, he suggests matching for mental age. The advantage of this is that differences in performance between well-nourished and malnourished children of the same mental age might turn up. This could then perhaps pinpoint specific changes induced by malnutrition and help to factor out the nutritional component from the other variables. Although this theory is certainly worth testing, the methods available at present for testing performance may limit its usefulness.

A final study should be mentioned (90), though it is still in progress and therefore cannot be critically evaluated. This is the study currently being conducted at INCAP. Aware of the inherent problems of control populations and of validity of testing, these investigators have set up a study in three villages. Great pains have been taken to assure that these villages have comparable populations. Food is supplied in one village, which is otherwise left alone; both food and social improvement are introduced into the second village; the third village is simply observed. The study has gone on for only a short time, but available data suggest that supplying food alone may significantly alter subsequent mental performance. However, preliminary data also suggest a marked sex difference in this response.

Although all these human studies clearly demonstrate retardation in development, they only implicate malnutrition as a cause. Although more work must be done, the data are, nevertheless, becoming more and more impressive when viewed as a whole. Each study has its own particular flaw, but since the flaws in each study are not the same, many of the variables cancel when the studies are combined. One common denominator seems to remain: severe early malnutrition.

Even if malnutrition is not the sole cause of the retarded development which has been described, the young infant is surely most at risk. We can certainly conclude that the complex of social ills encompassing severe malnutrition, which is prevalent throughout the world, affects the learning ability of infants. The infant population is not being protected adequately from what would appear to be permanent disability.

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THE ROLE OF AUSTRALIA ANTIGEN IN VIRAL HEPATITIS AND OTHER DISEASES[1]

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Australia antigen [Au(1)] is an infectious agent which can cause hepatitis in man. The data supporting the infectious hypothesis as well as the genetic, clinical, epidemiologic, and other studies have been reviewed elsewhere (1–4). Other current studies will be reviewed in this paper.

CHEMISTRY

The presence of genetic material in Au(1) was first reported by Jozwiak et al (5) in January, 1971. In their studies, nucleic acids were found in small quantities (5 percent) in purified Au(1), and colorimetric tests were negative for DNA. They concluded that Au(1) contains RNA; this has been confirmed by Millman and colleagues. In an earlier study, Millman et al (6) reported that if RNA were present in Au(1) it would constitute less than 10 percent of the total weight of protein.

Australia antigen has been isolated from plasma by pretreatment with a variety of proteolytic and other enzymes, followed by gel filtration and density centrifugation. The isolated particles of Au(1) (free of normal serum constituents) have an electron microscopic appearance similar to that of small viruses (Fig. 1a). When purified Au(1) is extracted with 2 percent Tween 80, the immunoreactivity to anti-Au(1) is destroyed. After this treatment, surface components are absent and coiled strands have appeared (Fig. 1b). This material appears to be mainly protein. The supernatant after Tween 80 treatment contains the following constituents (7): 1. IgG reacting with antisera to both heavy and light chain; 2. a component reacting with anti-beta 1a/1c (presumably C3 complement); 3. a component which reacts with anti-human beta-lipoprotein; 4. a component which reacts with anti-transferrin; and 5. a substance which reacts with anti-albumin. It thus appears that some of the components of Au(1) are substances similar to human serum proteins. The possibility of these representing serum proteins nonselectively absorbed on the surface is unlikely for the following

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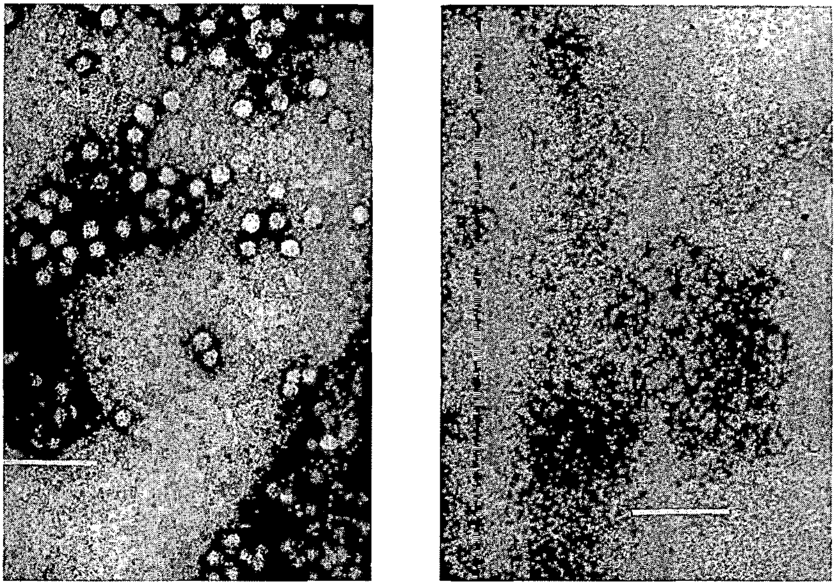

a b

FIGURE 1a. Negatively stained particles of purified Au(1) ×264,000. b. Negatively stained purified Au(1) particles following extraction with Tween 80 ×264,000.

reasons. 1. The preparations were treated with proteolytic enzymes during the purification procedure. 2. None of the components liberated by Tween 80 can be detected before Tween extraction. 3. The reactivity of Australia antigen is destroyed after treatment with Tween 80, indicating that the antigenic determinants for anti-Au(1) were removed or destroyed. It is unlikely that absorbed contaminants would contain the reactive sites for all anti-Au(1) antisera. 4. There is a difference in migration on immunoelectrophoresis of the IgG extracted after Tween treatment, and human serum IgG. 5. The beta-lipoprotein extracted with Tween 80 does not have cross-reactivity with normal human serum lipoproteins. 6. These results have been repeated with four separate preparations.

The absorption profiles of purified Au(1), the Tween 80 extract, and a phenol extract of the Tween 80 extract are shown in Figure 2. An absorption peak at 260 nm is seen with the phenol extract. Even the unextracted purified Au(1) demonstrates a small hump at 260 nm. This material produced a positive Orcinol reaction and negative diphenylamine reaction, and this is consistent with the presence of RNA.

TISSUE CULTURE

The presence of nucleic acid suggests that Au(1) is capable of replicating. Additional evidence for this comes from tissue culture studies. Coyne et al (8) studied tissue from 68 patients who had had liver biopsies for diagnostic

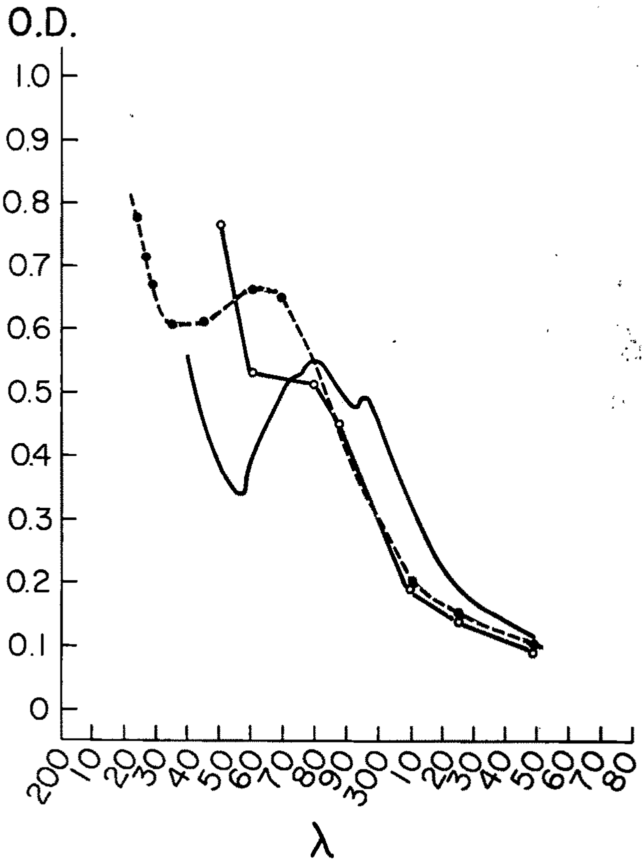

FIGURE 2. Absorption profiles of purified Au(1) (solid line), Tween 80 extract of purified Au(1) (line with open circles), and phenol extract of Tween 80 extract (dashed line).

purposes. Specimens from 40 patients were trypsinized, minced, and cultured on polyethylene discs. All cultures produced viable cells; Au(1) was found by radioimmunoassay in tissue culture fluids from one specimen. The other 28 were cultured without trypsinization; one produced an epithelioid cell culture. This was from a patient who had acute hepatitis with Au(1) in his blood. Fluorescent staining of the original biopsy specimen showed fluorescent granules in the cell nuclei. The culture obtained from this biopsy was maintained through 16 passages without obvious microscopic change in the appearance of the cells. Fluorescent granules were seen in the nuclei during the second and sixth passages (Figure 3). Cells from passage 6 were also grown on 32 polyethylene discs. Australia antigen was detected in fluids from two of the 32 discs by radioimmunoassay.

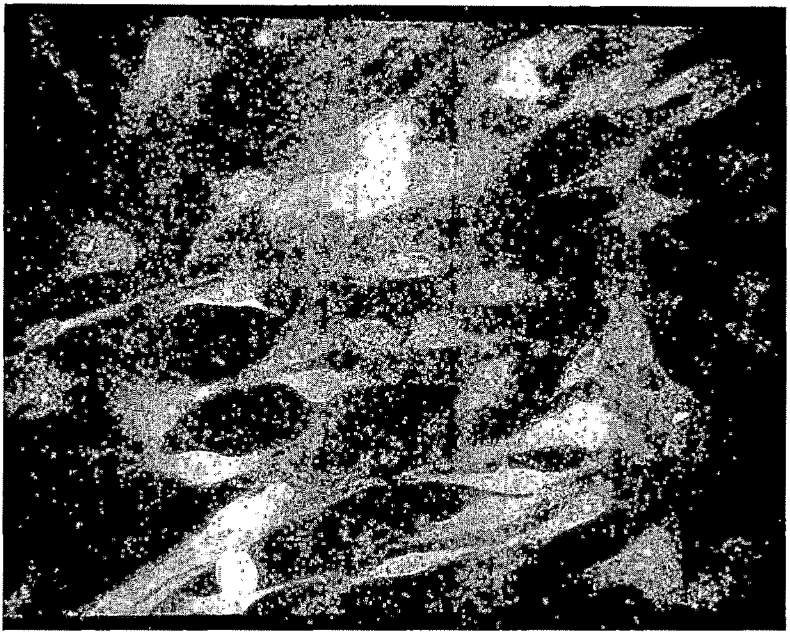

FIGURE 3. Fluorescent granules [after coating with fluorescein-labeled anti-Au(1)] in nuclei of cells cultured from liver biopsy specimen after six passages.

These studies demonstrate evidence of Au(1) by immunofluorescence in later generations of liver cells in culture, with apparent replication of Au(1) and release into the tissue culture fluid.

PERSISTENT AUSTRALIA ANTIGEN

Individuals who develop persistent Au(1) with clinically inapparent hepatitis, or with no evidence of hepatitis at all, have been of particular interest. These are potentially infectious, asymptomatic carriers of Au(1), and pose a hazard to the general population. We have proposed the hypothesis that they have an innate (perhaps inherited) susceptibility to developing this persistence, and can currently be detected only following exposure to the infecting agent. Persistent Au(1) is associated with certain specific groups of individuals:

1. Patients with chronic forms of hepatitis (ca. 20 percent) (9, 10).
2. Patients with Down's syndrome in large institutions for the mentally retarded (ca. 30 percent) (11, 12). In these patients persistence has been documented for 3–5 years. We have not seen Au(1) disappear in any of the Down's syndrome patients we have followed (1, 3, 13).
3. Patients with lymphocytic leukemia in the United States who have been transfused (ca. 15 percent) (14).

4. Patients with lepromatous leprosy (but not tuberculoid leprosy) (15, 16).
5. Patients with chronic renal disease who are the subjects of chronic hemodialysis programs (17).
6. Normal people in some populations (up to 20 percent, particularly in tropical areas) (1, 18, 19).

In the United States it occurs in a frequency of 0.1 percent in the general population; in certain groups (multiply transfused patients and drug abusers) it is found in a frequency of about 4 percent (20).

It is likely that the predisposition of these individuals to persistent infection may extend to organisms other than Au(1) (including *M. leprae*, the agent(s) responsible for leukemia, and others). This has been referred to as the "Australia affinity group" of agents (4).

There have now been a number of studies which may relate to this susceptibility to persistent infection. One concerns its inheritance; this can be best studied in parts of the world where the Au(1) agent is widespread in the environment. The susceptible individuals would be generally exposed, and would actually have persistent Au(1) at the time they were studied.

On the island of Cebu in the Philippines, Au(1) was found to cluster in families (21). Examination of the kindreds revealed that the distribution of individuals with Au(1) was not compatible with dominant inheritance, and a hypothesis of recessive inheritance was made. This hypothesis states that there is a gene (Au^1) which, when present in double dose, is responsible for susceptibility to persistence of Australia antigen. Individuals heterozygous for the gene or homozygous for the alternate allele would not have this susceptibility. Families were identified by having at least one positive offspring and, according to the hypothesis, each parent would have at least one Au^1 gene. By segregation analysis techniques, the total number of children expected to be positive was calculated, and then the number of offspring with persistent Australia antigen in the families studied was counted. The findings were very close to the predicted number supporting the genetic hypothesis (Table 1).

The same study was performed on the island of Bougainville in the Trust Territory of Papua and New Guinea with the same results (22), thus confirming the first genetic study. On the basis of these two independent studies, it is likely that susceptibility to persistence of Australia antigen is inherited as an autosomal recessive trait. Further confirmation was obtained during the past year from the laboratory of Ceppellini and his colleagues (23), who found persistent Australia antigen in a frequency of 10 percent among the Sardinian population living in Turin. A similar family study of this population yielded data which support the genetic hypothesis.

Investigation of possible factors responsible for this inherited susceptibility to persistence have been carried out on patients with Down's syndrome, an accessible population which is known to be susceptible to persistent

TABLE 1. INHERITED SUSCEPTIBILITY TO CHRONIC INFECTION WITH THE AU(1) AGENT: SUMMARY OF CALCULATIONS BY SMITH'S METHOD FOR CEBU AND BOUGAINVILLE FAMILIES

Location	Type of mating	No. of families	Number of recessive children		
			Observed	Expected	Variance
Cebu	Au(0)×Au(0)[a]	24	33	32.705	8.069
Cebu	Au(1)×Au(0)	7	12	12.189	3.556
Bougainville	Au(0)×Au(0)	41	56	54.815	11.583
Bougainville	Au(1)×Au(0)	24	48	42.590	12.675

[a] Phenotype Au(0) indicates that Australia antigen was not detected in the individual's blood. In this table parents with phenotype Au(0) are assumed to have genotype Au^1/Au by virtue of having at least one child with Australia antigen in his blood. Such a child is denoted by this hypothesis to have phenotype Au(1) and genotype Au^1/Au^1. An extensive discussion of this genetic hypothesis is found in reference 22.

Au(1) (11–13) (Table 2). A known high incidence of leukemia (24) also relates them to the "affinity group" hypothesis. (*a*) In short-term culture in the presence of phytohemagglutinin, lymphocytes from these patients do not synthesize DNA as readily as do lymphocytes from control subjects (25). This was determined by measuring their DNA polymerase activity and the incorporation of tritiated thymidine into the cells. (*b*) They also have a diminished skin reactivity to a panel of antigens, indicating an impaired cellular immune response (26); this might be related to the defect in their small lymphocytes. (*c*) There are also abnormalities in their im-

TABLE 2. PATTERN OF IMMUNOLOGIC AND HEMATOLOGIC RESPONSE IN PATIENTS WITH DOWN'S SYNDROME

Diminished

- ↓ Lymphocyte function
  - ↓ DNA polymerase activity
  - ↓ ^{3}H-thymidine incorporation
- ↓ Skin reactivity
- ↓ γM level

Exaggerated

- ↑ γG response
- ↑ Basophils
- ↑ Response to epinephrine
  - ↑ Leukocytosis
  - ↑ Hyperglycemia

munoglobulins (27). They have an innate deficiency of IgM. When they are exposed to the environmental factors of large institutions for the mentally retarded, they have a prolonged elevated IgG response. This might be a "compensatory" response of their humoral immune mechanisms for the impairment in cellular immunity. (*d*) These patients also have a significantly higher number of basophils in their blood than do controls (26). Basophils have been related to the immune response. (*e*) They also have an apparently excessive response to stressful stimuli (26). This was measured by the degree of leukocytosis and hyperglycemia following epinephrine injection, which was significantly higher than the control group. This type of response might also be considered as a "compensatory" phenomenon.

A high frequency of peristent Au(1) can be detected only when there is exposure to this agent. This has been shown in several different ways (Table 3). 1. There is a high frequency of persistent (Au(1) in patients with Down's syndrome in large state-supported institutions for the mentally retarded, while the frequency in small private institutions and in patients who live at home is quite low (12, 13). 2. Persistence of Au(1) in lepromatous leprosy patients can be demonstrated in leprosaria in countries with a generally higher frequency of the antigen in the population (15, 16), but not in countries with a low frequency (28). 3. The frequency of persistent Au(1) in transfused leukemia patients is quite high in the United States, where commercial blood is commonly used (11, 14, 18); it is absent in Canada, Denmark, and Finland where commercial sources of blood are not used (29). 4. In the United States, a higher frequency of Au(1) is found in those individuals exposed to multiple blood transfusions or to parenteral drug administration (drug abusers) than in the general population not so exposed (20).

A survey of the sex frequency in 23 populations and disease groups revealed a consistently higher frequency of Australia antigen in males than in females (30) (Table 4). A review of studies from other laboratories in which sex frequency was included revealed similar findings (23, 31–38) (Table 5). Previous studies have indicated that males have a generally higher susceptibility to infection than females.

In diseases characterized by an increased production of antibody, there is a higher frequency of females than males. The sex distribution of Au(1) is consistent with the hypothesis that females have more active immunologic responses than do males.

There may be groups of individuals who have a similar relationship to persistent antibody to the antigen. This has been demonstrated in a group of 169 patients with thalassemia (39) of whom 18 had persistent Au(1) and 35 had persistent antibody to the antigen. Those who had antigen detectable at one time always have the antigen and those who had antibody detected at one time always have the antibody. Those patients with Au(1) had an increased mortality when compared with those with persistent antibody or those without antigen or antibody. Apparently a defined group of indi-

TABLE 3. OCCURRENCE OF PERSISTENT AUSTRALIA ANTIGEN IN SUSCEPTIBLE PEOPLE INTIMATELY EXPOSED AND NOT EXPOSED

	Frequency of Au(1) in									
	Heavily exposed			Source of Exposure	Little or no exposure				χ^2	p
	N	Au(1)	%		N	Au(1)	%			
Leukemia	293	37	12.6	Commercial blood	103	0	0	Volunteer blood	11.23	$.803 \times 10^{-3}$
Down's syndrome	310	86	27.7	Large crowded institutions	45	0	0	Live at home	10.792	$.102 \times 10^{-2}$
Lepromatous Leprosy	1359	115	8.5	Au(1) in general population	158	0	0	Au(1) rare in general population	12.097	$.505 \times 10^{-3}$
Normal	476	19	4.0	Commercial blood (Patients) Contaminated needles (Drug abusers)	4147	8	0.2	General U. S. population	95.62	1×10^{-9}

TABLE 4. Sex Ratio of Australia Antigen in Patients With Various Diseases (A) and Normal, Nonhospitalized Populations (B)

A. Disease	Male		Female		Total		Ratio
	N	%Au(1)	N	%Au(1)	N	%Au(1)	
Acute viral hepatitis	112	35.7	94	19.1	206	28.2	1.87
"Chronic active hepatitis"	12	25.0	19	0	31	9.7	[c]
Down's syndrome	212	31.6	92	18.5	304	27.6	1.88
Other mentally retarded	145	3.4	43	2.3	188	3.2	1.48
Leukemia	173	12.7	124	8.8	297	11.1	1.44
Lepromatous leprosy (Cebu)	396	11.4	188	5.3	584	9.4	2.15
Lepromatous leprosy (India)	451	7.1	101	2.0	552	6.2	3.55
Tuberculoid leprosy (Cebu)	343	5.5	262	2.7	605	4.3	2.03
Tuberculoid leprosy (India)	281	2.8	103	0	384	2.1	[c]
Chronic hemodialysis	5	100.0	4	75.0	9	88.9	1.33
$\chi^2=42.3270$		P=.00251					

B. Population	Male		Female		Total		Ratio
	N	%Au(1)	N	%Au(1)	N	%Au(1)	
Marshall Islands	243	7.8	226	6.2	469	3.3	1.26
Cebu, Philippines	678	6.9	609	2.8	1287	5.0	2.46
Cashinahua, Peru	45	22.2	44	13.6	89	18.0	1.63
Manila	138	4.3	59	5.1	197	4.6	0.84
Polynesia	597	1.3	550	0.54	1147	0.95	2.41
New Hebrides	597	10.2	394	4.8	991	8.1	2.13
New Caledonia	518	2.50	444	1.6	962	2.1	1.56
India (South)	116	4.3	135	0	251	2.0	[c]
British Solomon Islands	127	5.5	160	2.5	287	3.8	2.20
New Guinea	532	3.0	287	1.7	819	2.6	1.76
Micronesia	330	3.9	292	2.1	622	3.1	1.86
Surinam	310	2.25	320	1.56	630	1.90	1.44
Bougainville	931	12.03	906	10.7	1837	11.37	1.12
$\chi^2=47.6840$		P=.0058					

[c] Au(1) not detected in the females.

viduals have a greater potential for developing adequate antibody when exposed to an antigen.

Furthermore, the anti-Au(1) in these patients is associated with antibodies against Gm, an antigen on IgG immunoglobulin (Table 6) but not with antibodies against other serum factors. Since Gm types are known inherited traits, the presence of anti-Gm must also be related to genetic factors, in much the same way that antibodies against a red blood cell antigen (e.g., anti-A, anti-B) develop only in an individual who has not inherited

TABLE 5. Sex Ratio of Australia Antigen in Patients with Various Diseases (A) and Normal Nonhospitalized Populations (B) Reported by Other Laboratories

A. Disease	Male N	Male N Au(1)	Male % Au(1)	Female N	Female N Au(1)	Female % Au(1)	Ratio	P
Chronic hepatitis (31)	22	4	18.2	13	2	15.4	1.18	.801
Down's syndrome (32)	21	3	14.3	9	0	0	[c]	.595
Down's syndrome (33)	70	31	44.3	64	16	25.0	1.77	.031
Other mentally retarded (33)	70	15	21.4	64	8	12.5	1.71	.254
Renal transplant patients (34)	33	13	39.4	34	1	2.9	13.4	.00076
Dialysis patients (34)	48	15	31.3	40	5	12.5	2.50	.067
Various diseases (35)	~6000	27	.45	~6000	14	.23	1.93	.061
Acute hepatitis (36)	16	7	43.8	11	4	36.4	1.20	.99
		$\chi=36.5549$			$P=.00242$			

B. Population	Male N	Male N Au(1)	Male % Au(1)	Female N	Female N Au(1)	Female % Au(1)	Ratio	P
Sardinia (23)	672	57	8.4	451	32	7.2	1.20	.0056
Japan (37)	4222	52	1.23	1024	2	0.20	6.31	.0091
Japan (38)	3830	89	2.32	1409	16	1.14	2.05	.465
		$\chi^2=21.3904$			$P=.00162$			

[c] Au(1) detected only in males.

that antigen. These findings provide further evidence of inheritance of susceptibility to persistent Au(1), and of the development of anti-Au(1). When specific Gm types are studied in these patients (Table 7), those with anti-Au(1) have a lower frequency of each Gm type than do those with persistent Au(1). This is true in every one of the seven comparisons made.

HEPATOMA

There is some reason to suspect that malignant hepatoma might be related to Au(1) (40). It is most common in Africa and Asia where high frequencies of the antigen have been reported in the general population (1, 18,

TABLE 6. Anti-Gm in Thalassemia Patients

	N	Anti-Gm	%	Probability	
Anti-Au(1)	30	22	73.3	$\chi^2=5.3892$	$p=.0203$
Au(0)	76	35	46.1	$\chi^2=6.8606$	$p=.0088$
Au(1)	17	5	29.4		
Total	123	62	50.4		

TABLE 7. GM TYPES IN THALASSEMIA PATIENTS

	Au(1) (N=11)		Anti-Au(1) (N=24)	
	+	%	+	%
Gm(1)	7	63.6	9	37.5
Gm(2)	1	9.1	1	4.2
Gm(4)	11	100.0	21	87.5
Gm(5)	10	90.9	21	87.5
Gm(10)	10	90.9	21	87.5
Gm(11)	10	90.9	20	83.3
Gm(21)	7	63.6	8	33.3

19). It is often found to develop in livers with a background of chronic hepatitis and postnecrotic cirrhosis. We might look upon the possible role of Au(1) in hepatoma in the context of the presumed pathogenesis of this tumor (Figure 4). Patients who are infected with Au(1) may actually get acute hepatitis or only transient Au(1) without evidence of hepatitis. The hepatitis may be clinical or subclinical, and may subside with complete recovery. Some patients with acute hepatitis develop chronic hepatitis, and some patients who get only Au(1) become chronic carriers of Au(1) without the development of chronic hepatitis. Chronic hepatitis results in damage to the liver; we do not know the effects of persistent Au(1) (without hepatitis) on the liver. It is possible that liver damage may result from this as well. Postnecrotic cirrhosis may follow, and it is either this cirrhosis or the associated liver damage which eventuates in the development of malignant hepatoma in some patients. This chain of events involves selection of patients throughout. Not all those infected develop acute disease. Only a few of these subsequently develop chronic hepatitis. The development of postnecrotic cirrhosis and hepatoma involves further selective factors.

Initial observations on the frequency of Australia antigen in hepatoma patients did not reveal any significant association (41). However, there are reports suggesting a strong association in some parts of the woild (42–49) (Table 8). There are now a number of reports of the familial co-occurrence of hepatoma and Australia antigen. Ohbayashi, Mayumi & Okochi (50)

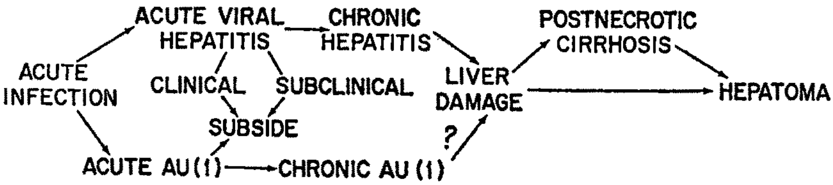

FIGURE 4. Hypothetical development of malignant hepatoma (see text).

TABLE 8. AUSTRALIA ANTIGEN IN HEPATOMA

Country	Hepatoma Patients			Controls			χ^2	p
	N	Au(1)	%	N	Au(1)	%		
U.S.A. (41)	55	2	3.6	55,956	58	0.1	35.3207	$.288 \times 10^{-8}$
U.S.A. (42)	35	0	0	—	—	—	—	—
U.S.A. (43)	8	5	62.5	—	—	—	—	—
U.S.A. (40)	12	0	0	2,412	2	0.1		N.S.
U.S.A. (44)	9	1	11.1	—	—	—		
East Africa (40)	11	0	0	512	8	1.5		N.S.
Uganda (41)	34	4	11.8	311	6	1.9	7.3294	.00678
Uganda (45)	45	18	40.0	122	4	3.3	35.6117	$.257 \times 10^{-8}$
Senegal (41)	210	88	42.0	1,160	140	12.1	111.9559	$.2 \times 10^{-9}$
Greece (46)	13	4	30.8	—	—	—		
England (47)	33	2	6.1	—	—	—		
Hong Kong (40)	42	2	4.7	50	2	4.0		
Chile (48)	5	3	60.0	1,617	8	0.5	(Fisher test)	$.23 \times 10^{-5}$
Total	512	129	25.2	62,140	228	0.4		

reported two such families, and have informed us of three additional ones (51). These families included many patients with hepatoma, other liver diseases, and persistent Au(1) without disease. Denison, Peters & Reynolds (44) reported a family in which there were three patients with hepatoma; the one available for testing had Au(1). An additional family with multiple liver diseases and chronic carriers was reported by Bancroft et al (52).

These data are consistent with an hypothesis similar to that which we have presented previously (4, 14). According to this hypothesis, there is a group of individuals that is susceptible to the development of persistent Au(1) after they are infected. These people are also susceptible to persistence of other infectious (and noninfectious) agents. This susceptibility to persistence may be an inherited trait as suggested by the previous family studies. If these susceptible individuals are exposed to the agent (or agents) causing malignant hepatoma, they will develop this disease as well. Such an agent may be common in some populations, but uncommon or rare in other parts of the world. According to this view, Au(1) would not necessarily be an etiologic agent for hepatoma, but persistent Au(1) would be a marker for an individual who would be susceptible to the development of the malignant disease if exposed to the appropriate agents.

TECHNIQUES, SPECIFICITIES, CLINICAL USES

For research purposes, and perhaps for most clinical purposes, double diffusion in agar gel is still preferred by many (53). This is the only technique which permits detection of immunologic identity with a known serum containing Au(1) and may permit the observation of different specificities. When done carefully by the prescribed technique, it is sensitive and easy to interpret. A variety of other techniques has been introduced since then, including counter immunoelectrophoresis, complement fixation, radioimmunoassay, hemagglutination, and platelet inhibition. All have the disadvantage of not permitting determination of identity with a standard, but some can be performed more rapidly than double immunodiffusion, and some may be more sensitive. The most sensitive appears to be the radioimmunoprecipitation assay. This technique is particularly sensitive for the detection of antibody, and will be very useful for future studies relating to the development of anti-Au(1).

There is now developing some interest in the possible multiplicity of determinants of Au(1) and possible specificities of antibody to Au(1). This was first pointed out by the studies of Levene & Blumberg (54) demonstrating mutiple specificities of rabbit antisera to the antigen. Subsequent studies by Raunio and colleagues (55, 56) have indicated that similar differences in specificities occur between human antisera. These findings could not distinguish between differences in antigenic determinants of the antigen, or differences in the abilities of individuals to develop antibodies against different antigenic determinants, thus resulting in different antibody specificities. Several other laboratories have now begun to report differences in human antisera (57–59), suggesting that different subtypes of Au(1) may occur. Further work on this subject is now in progress.

The test for Au(1) has several well-established clinical applications, and may be useful for other purposes in the future (60). The most obvious use is in the screening of donor blood for transfusion. It is generally agreed that removal of blood containing the antigen from the blood bank results in a significant decrease in the amount of post-transfusion hepatitis in the recipients. We have been studying post-transfusion hepatitis at a large urban hospital (Philadelphia General Hospital) (Table 9). The incidence of hepatitis (icteric or anicteric) in transfused patients was the same in 1968–1969 (18 percent) as in a previous study in 1963–1964 (61). Beginning in 1970, all Au(1)-containing blood was effectively excluded from use by the blood bank. The incidence of hepatitis fell dramatically to 3.3 percent (62). The test can be used for the diagnosis of acute hepatitis and chronic forms of

TABLE 9. FREQUENCY OF POST-TRANSFUSION HEPATITIS AT PHILADELPHIA GENERAL HOSPITAL

Year	Recipients followed	Hepatitis	%	
1964–65	56	10	17.9	
1968–69	78	14	17.9	p = .0057
1970	61	2	3.3	

hepatitis when these diagnoses are obscure. In this case a positive test for Au(1) strongly supports the diagnosis while a negative test does not exclude it. The test is also useful in determining progression from acute to chronic hepatitis. If a patient with acute hepatitis has Au(1) which persists for longer than 4–6 weeks, it is likely that it will persist for months or years and he will develop a chronic form of hepatitis. On the basis of the study of the antigen in patients with thalassemia, the test may be useful in determining the prognosis of these patients. Those with Au(1) tend to have a poor prognosis. The test can also be used in the epidemiologic surveillance of various institutions. It has proven of value in institutions for the mentally retarded, and has detected potential sources of infection in these institutions. The widespread use of such surveillance techniques might lead to a revision of the methods of managing such institutions, with the consideration of separating those who are chronic carriers or possibly the distribution of patients into smaller units. The test for Au(1) has been of importance in surveillance of a research laboratory dealing with the antigen in human blood and tissues (63). It has permitted early detection of patients who might be developing hepatitis, and may have been of value in preventing the development of more severe disease. It might be considered for surveillance of other high risk groups including those in clinical laboratories, blood banks, and blood donor stations, pathology laboratories, morgue personnel, and the staff of renal dialysis units. It would also be of value in the periodic testing of possible sources of infection outside of institutions, including patients in chronic hemodialysis units, food handlers, and perhaps dentists and dental assistants.

Some of these uses of the Au(1) test, particularly the testing of blood donors and the diagnosis of hepatitis, have already been instituted in many parts of the world, and it is likely that this will be virtually universal in the not too distant future. The value of other potential uses of the Au(1) test, and the possible application of different specificities of antisera, are yet to be determined.

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NEUTROPHIL PROTEASES IN INFLAMMATION 7036

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INTRODUCTION

It has long been recognized that polymorphonuclear leukocytes (PMN) entering an acute inflammatory exudate can be injurious to tissues in addition to serving a beneficial role through phagocytosis of immune reactants or microorganisms. A classic proposal in this field (1) was that some of the agents responsible for intracellular digestion of phagocytized particles might also damage host tissues if released extracellularly in large enough quantities. Much of the older information dealing with this subject has already been reviewed by Hirsch (2), Thomas (3), Weissmann (4), Cochrane (5–7), Barnhart et al (8), Hersh & Bodey (9), Janoff (10), and Cline (11). It is the purpose of the present article to summarize more recent findings concerning the mediation of tissue damage by one class of agents present in PMN, namely proteases.

PMN ACID CATHEPSINS

The acid proteases of PMN have been suggested as prime mediators of tissue damage in inflammation. In addition to a potentially direct action on tissue targets, PMN-acid cathepsins may also play a part in inflammation indirectly via kinin generation (12, 13). Rabbit PMN acid cathepsins have been extensively studied by Cohn & Hirsch (14), Lapresle & Webb (15), Wasi et al (16), and Cochrane & Aiken (17). The latter workers also investigated the capacity of rabbit PMN acid cathepsins to degrade vascular basement membranes. Their results showed in vitro digestion of glomerular basement membrane by rabbit PMN granules, with optimal effects in the acid pH range. Two responsible enzymes were isolated and proved to have physicochemical properties similar to those of cathepsins D and E, with maximum activity at pH 3.4 and 2.5, respectively. Little or no in vitro activity against basement membrane was detected at neutral pH in their experiments and, as expected, introduction of the rabbit PMN acid cathepsin fractions into living tissue failed to induce detectable basement membrane damage in vivo. On the other hand, basement membrane damage was observed in vivo following accumulation of PMN at Arthus sites or after local injection of antibody to vascular basement membrane. The authors therefore suggested that

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lowered tissue pH, resulting from lactic acid production by PMN, was an obligatory precondition for acid cathepsin activity in vivo. However, in evaluating their conclusions, it is worth noting that the formation of complexes between trypsin and its principal endogenous serum inhibitor, alpha$_1$ antitrypsin (A_1AT) is maximal at neutral pH but decreases rapidly below pH 5.0 (18). Therefore, a diminished effectiveness of the major endogenous protease inhibitor would be a likely additional consequence of lowered tissue pH. Since A_1AT suppresses neutral protease activity of PMN (19), facilitation of tissue damage by PMN lactate production may not depend exclusively upon the acid cathepsins.

Clearly, the delineation of the pH at target sites in inflamed tissue (e.g., at the interface between PMN and vascular basement membrane) remains a question of fundamental importance. Measurement of this pH has not yet been accomplished. Although earlier experimental observations of Opie (20) and Menkin (21) had shown an alkaline pH of PMN-rich, acute inflammatory exudates, these conditions may not be present within tissues in the area of immediate proximity to the leukocytes. For example, PMN-induced damage at Arthus sites may depend on the intimacy shared by crowds of PMN and components of the vessel wall, where a concentration of acid sufficient to damage cells directly or to permit acid cathepsin action may be produced. In their studies of the local Shwartzman phenomenon, Thomas & Stetson (22) measured an increased release of lactic acid in endotoxin "prepared" skin sites that were found to be rich in PMN. Similar results were found in analysis of Arthus sites removed from rabbits 1–3 hours after initiation of the reaction (23). To demonstrate the importance of PMN in lactate production, rabbits could be depleted of these cells with HN_2, and no increase in lactate production occurred upon local injection of endotoxin (24).

On the other hand, the neutral PMN proteases may be mainly responsible for extracellular damage, while the acid PMN proteases may function primarily in intracellular digestion of phagocytized protein. It is likely that within the confines of cytoplasmic vacuoles, the concentrations of hydrogen ion required for acid cathepsin activity are more readily attained, although this was not substantiated in recent experiments with human PMN (25).

PMN NEUTRAL PROTEASES

In contrast to rabbit leukocyte fractions, granule extracts from human PMN were shown to digest vascular basement membrane at physiologic pH in vitro and to damage this structure in vivo (26, 27). These observations were in keeping with an extensive series of older studies, which had shown that PMN of humans and of most species except the rabbit, contain strong proteolytic activity at physiologic or slightly alkaline pH (28, 29). More recently, protein-polysaccharide-cleaving activity has been detected in rabbit PMN granules at both neutral and acid pH (30), as well as a neutral histone-digesting enzyme (31, 32). Thus, rabbit PMN are probably not entirely devoid of potentially tissue-damaging neutral proteases, and the mediation of basement membrane damage in Arthus reactions in rabbits may be due to the action of these enzymes in addition to the acid cathepsins.

Neutral protease activity of PMN has been demonstrated in a variety of species and against a broad range of substrates. However, it is not clear at present how many different neutral proteolytic enzymes may contribute to the foregoing spectrum of activities, or to what extent the PMN content of selected neutral proteases may vary from one species to the next. Several apparently distinct neutral enzymes have been partially characterized. These include fibrinolytic factors isolated from human PMN (33–35), the collagenase and the elastase from human PMN (discussed later), the histone-degrading enzyme of rabbit PMN (31, 32), and several low molecular weight neutral proteases (cathepsins F and G) from porcine PMN (36). Of these, only a few have been selected for further discussion.

HUMAN GRANULOCYTE FIBRINOLYSINS

Degradation of fibrin by intact, human PMN leukocytes and by proteolytic extracts of these cells was described by Barnhart et al (8). Their experiments showed that eosinophils, especially, were rich in a neutral, profibrinolytic enzyme. Prokopowicz and associates were able to demonstrate proteases with fibrinolytic activity equally well in neutrophils. Three neutrophil enzymes were described by these workers. One was shown to have spontaneous fibrinolytic activity which could be increased by removal of an inhibitor present in the leukocyte homogenate (33). The second enzyme was found primarily in an inactive form and appeared to be identical to the profibrinolysin of plasma (34). The leukocyte plasminogen was separable from the spontaneous fibrinolysin, since the former enzyme was retarded by anion exchanger at alkaline pH, whereas the latter was not (35). When activated, granulocyte plasminogen showed marked esterolytic activity against synthetic trypsin substrates such as tosyl arginine and lysine methyl esters. (Of these fibrinolytic enzymes, the spontaneous fibrinolysin most resembles PMN elastase,—one of the other neutral proteases to be discussed later. For example, both activities are partly inhibited by a factor present in the leukocyte homogenate, neither is retarded by anion exchanger at alkaline pH, and neither hydrolyzes synthetic trypsin substrates.) The third factor described by Prokopowicz possessed plasminogen activator activity. All three proteases of the PMN fibrinolytic system appeared to be evenly distributed among the different subcellular fractions of leukocytes (33), a finding which differs from the experience of other workers with the PMN collagenase and elastase.

HUMAN GRANULOCYTE COLLAGENASE

In the first of two papers dealing with this factor, Lazarus et al (37) provided evidence of collagenolytic activity (operative at neutral and alkaline pH) in human granulocytes and showed that the enzyme was primarily recoverable from the lysosome fraction of the cells. Human lymphocytes were inactive. Using a reconstituted collagen fibril gel prepared from native, acid-soluble skin collagen of rats injected with ^{14}C-glycine, they demonstrated solubilization of labeled digestion products by human PMN granule extracts. Their substrate, moreover, was almost completely resistant to nonspecific digestion by trypsin. Collagenolytic activity was abolished in the presence of 0.01 *M* sodium ethylenediaminetetraacetate (EDTA), and most of the activity was lost below pH 6.5. Collagenolytic activity of human PMN granules was also demonstrated by viscosity measurements of enzyme-treated collagen solutions.

The granulocyte collagenase was partly purified by chromatography on diethylaminoethyl cellulose (DEAE) to which the enzyme was bound. This purified preparation gave evidence of specific collagenolysis when its digestion products were analyzed by acrylamide gel electrophoresis. After precipitation of these products in concentrated salt and re-solution in 8 *M* urea, their electrophoretic behavior was characteristic of discrete units of low molecular weight similar to those seen after treatment of collagen with other specific animal collagenases. Evidence of the diffuse peptide front frequently found after nonspecific proteolysis was not apparent. This suggested that the purified granulocyte collagenase resembled the specific collagenases obtained from other vertebrate tissues.

Subsequently, Lazarus et al (38) reported additional properties of the partially purified granulocyte collagenase. They showed that this enzyme was not only inhibited by 0.01 *M* EDTA as mentioned above, but also by 0.01 *M* cysteine or reduced glutathione. Specific cleavage of collagen monomers by the purified enzyme was not inhibited by serum. This represents a marked difference between granulocyte collagenase and granulocyte elastase, since the latter enzyme is inhibited by serum but not by 0.01 *M* EDTA (see below). Electron microscopy was carried out on segment-long-spacing aggregates of collagen prepared from solutions of substrate treated with the purified granulocyte enzyme. These experiments revealed that collagen monomers were cleaved into $\frac{3}{4}$ and $\frac{1}{4}$ length fragments, and provided further support for the view that the site of action of granulocyte collagenase was similar to that of other specific vertebrate collagenases. The cleavage point appeared to be $\frac{1}{4}$ of the length from the carboxy-terminal end of the molecule.

Of considerable interest was Lazarus's observation that specific cleavage products of collagen breakdown by the purified leukocyte enzyme could be further degraded by another protease present in crude extracts of human PMN granules and recovered in the fraction which was not retarded by DEAE at alkaline pH. The second enzyme appeared to be nonspecific in its action on collagen and its activity was inhibited by serum. In all of these respects the second protease resembled the PMN elastase to be discussed next. In the absence of supplementary digestive activity, physical dissolution of collagen fibrils by purified granulocyte collagenase was relatively inefficient. As pointed out by Lazarus, PMN collagenase (or more properly the enzyme system composed of the specific collagenase and the broadly acting second protease) "may well be involved in collagen destruction in a wide spectrum of pathologic states (including) acute inflammation, abscess formation, and certain immunologic phenomena such as Arthus vasculitis, serum sickness vasculitis, and nephrotoxic nephritis."

HUMAN GRANULOCYTE ELASTASE

Human PMN also contain an elastin-degrading enzyme (39), which can be separated from the collagenase described above by ion exchange chromatography (27). The elastase is maximally operative at about pH 8.5 but retains considerable activity at lower pH values. Its activity was measured initially with orcein-impregnated elastin from bovine ligamentum nuchae as substrate. Later, several synthetic substrates for elastase were included in the experiments, and were found to be readily hydrolyzed by the human granulocyte enzyme (40, 41). Like granulocyte collagenase, the elastinolytic protease is also primarily present in the granule (lysosome) fraction of PMN (39). Granule extracts prepared from lymphocytes contained little elastinolytic activity (39), but recent studies have detected weak elastinolytic activity in human (42) and rabbit (42, 43) alveolar macrophages and human platelets (44, 45).

Human granulocyte elastase possesses the following properties: It is partly inhibited by trypsin-inhibitor from soybean and salivary gland and is completely inhibited by the levels of anti-elastase present in a 10–20 percent concentration of serum. More recent observations (46) implicate alpha$_1$antitrypsin as the principal serum inhibitor of the leukocyte elastase. Pancreatic elastase, on the other hand, is not inhibited by equivalent amounts of soybean and salivary antitrypsin but is 10-fold more sensitive to serum inhibition (1–2 percent serum is effective). Pancreatic and PMN elastases also differ significantly with respect to salt inhibition and inhibition by lowered pH. Moreover, granulocyte elastase resists inhibition by EDTA at concentrations which largely abolish the activity of granulocyte collagenase or of pancreatic elastase (27). Leukocyte elastase is destroyed by heating to 100°C. Increasing concentrations of granule protein or length of incubation time produce linear increases in elastolysis only up to the point where an as yet unidentified portion of the elastin fiber, serving as substrate for the enzyme, is exhausted. This point is reached well before complete solubilization of the fiber (Loeven, personal communication).

Staining of arterial elastic fibers in frozen sections of human kidney and in segments of human renal arteries is decreased after incubation of the tissues with human PMN granule extract in vitro (39). Intramural injection of the PMN granule extract into dog abdominal aorta in vivo results in loss of elastic staining in the tunica media and adventitia of the vessel. The effect can be noted as early as 2 hours after injection (27).

Partial purification of granulocyte elastase has been achieved by chromatography of PMN granule extracts on DEAE cellulose followed by gel filtration through Sephadex G50 (27). Subsequent iso-electric focusing of the partly purified elastase fraction revealed further heterogeneity (41), in that four distinct peaks of protein were separated by the latter procedure. The elastase focused at the extreme alkaline end of the pH gradient. The heterogenous fraction obtained by column chromatography showed about 5-fold increased specific activity against both dyed elastin and synthetic substrates. A second chromatographic fraction which solubilized bovine Achilles' tendon collagen was also recovered from the DEAE column, using a modification of Lazarus's original method for purification of granulocyte collagenase. This fraction possessed no detectable activity on dyed elastin or synthetic substrates of elastase. When these two granule fractions were injected separately into rabbit skin, the elastase-enriched fraction produced the hemorrhagic lesion given by whole extracts of PMN granules with about 5-fold increased activity over that of the crude extract. The fraction with collagenase activity, but no elastase activity, produced no grossly detectable tissue damage at comparable doses of protein. Rabbit renal basement membrane incubated with these enzymes at neutral pH was well solubilized by the elastase-enriched fraction (15–20 percent after 16 hours incubation) but was only slightly solubilized (2–3 percent) by the fraction containing collagenolytic activity free of the other protease. These results suggest that human granulocyte collagenase, by itself, does not efficiently solubilize the collagen-like protein component of glomerular basement membrane, and that the elastolytic protease of PMN may be the second, nonspecific protease referred to by Lazarus as being obligatory for physical dissolution of collagen fibrils by the PMN protease system (38).

PMN fractions containing the elastase (but not those containing the collagenase separated from it) degraded protein-polysaccharide of rabbit articular (tibial) cartilage. Both chondroitin sulfate and protein were released from articular cartilage, and staining of the cartilage with toluidine blue after incubation revealed a marked loss of metachromasia (47). In this context it is interesting to note that the human granulocyte elastase is inhibited by the anti-rheumatic agent, gold sodium thiomalate (48). The possible role of this enzyme in the pathogenesis of human joint disease requires further study.

More recently, it has been shown that the digestion of denatured hemoglobin at pH 7 by whole extracts of human PMN granules is inhibited 50 percent by a 0.02 *M* concentration of *tert*-butyloxycarbonyl-L-alanine, an esterolysis product derived from a synthetic elastase substrate (41). Equivalent inhibition of tryptic digestion of hemoglobin can be achieved under the same conditions by using a 0.02 *M* concentration of tosyl arginine methyl ester, a synthetic ester substrate for trypsin, yet the esteratic trypsin substrate has no inhibitory effect upon hemoglobin breakdown by leukocyte granules. These data suggest that a major part of hemoglobin digestion by human PMN granules at pH 7 may be due to the action of the elastolytic protease. Such a conclusion is supported by the observation that elastases in general are rather nonspecific in their affinity for protein substrates, unlike collagenases which are highly selective in their effects. A considerable part of the neutral protease activity of human PMN granules may be due to the activity of the elastolytic enzyme. Accordingly, it seems likely that leukocyte elastase plays an important role in PMN-mediated tissue injury, not only involving elastin per se (as in serum sickness arteritis) but a variety of other connective tissue targets as well. Examples of the latter may be basement membrane in the Arthus vasculitis and in PMN-dependent forms of glomerulonephritis and cartilage matrix proteoglycan in certain joint disorders in which the synovial effusions are rich in PMN (as in septic and gouty arthritis). The recent demonstration of similar enzyme activity in human alveolar macrophages (42) focuses attention on the possible role of leukocyte elastases in the pathogenesis of emphysema (49). At the same time, these observations do not exclude the role of other neutral and acid proteases of PMN in the mediation of tissue damage.

TARGETS OF PMN PROTEASES IN VIVO

The preceding sections provided evidence of the digestion of connective tissue components such as vascular basement membrane, collagen, elastin, and cartilage matrix proteoglycan by PMN proteases in vitro. Evidence will now be outlined (primarily based on experimental models of immune-induced tissue injury) which suggests that some of these same tissue components also serve as targets of PMN-borne agents in vivo. In addition, some soluble constituents of serum appear to be important substrates for proteolytic attack during inflammation, including the enzymes originating in PMN. Two of these, kininogens and complement, will be included in the following discussion.

VASCULAR BASEMENT MEMBRANE

Vascular basement membrane constitutes a major barrier to the passage of macromolecular substrates from the blood stream into the interstitial fluid or urine. In Arthus reactions and nephrotoxic serum nephritis, in experimental animals, neutrophils are attracted to sites of deposition of immune reactants in the vessel wall which have often localized along the basement membrane. Alteration of the latter structure then follows, and macromolecules, red cells, etc., pass freely through the barrier (17). Basement membrane damage can also occur in inflammatory reactions incited by agents other than antigen-antibody complexes (50–54). The degradation of basement membrane, which appears to take place in the presence of neutrophils, has been monitored in vivo by a loss of antigenicity of the membrane and its structural fragmentation as shown by electron microscopy (17). These ultrastructural studies also show that neutrophils frequently gain intimate contact with the basement membrane by dissecting between this structure and the overlying endothelial cell. In glomerulonephritis, proteinuria is noted in conjunction with the ultrastructural evidence of basement membrane damage by PMN, and the urine can be shown to contain basement membrane fragments as well as large protein molecules which have cleared the filtering membrane (55). Specifically, during the neutrophil attack two basement membrane fragments, normally excreted in low concentration, appear in the urine in much greater concentration, and in addition, unique basement membrane fragments are also found. At the same time, large molecules such as IgG are cleared from the circulating plasma across the glomerular filter in relatively large amounts (55).

INTERNAL ELASTIC LAMINA

In the arteritis of experimental serum sickness, a key structure that becomes disrupted under the bombardment of PMN is the internal elastic lamina (56). The earliest lesion observed in this arteritis appears to be a lifting of the intimal cells away from the internal elastic lamina, leaving a fine space which often contains a few round cells. However, the elastic lamina remains intact and inflammation does not advance into the arterial media until neutrophils enter the site. Then marked disruption of the elastic lamina occurs and inflammatory change continues into the media. A role for leukocyte elastase in this reaction would appear to be an obvious possibility.

SERUM PROTEINS

Kininogens.—The capacity of leukocytes to catalyze the formation of kinins would be of obvious importance in influencing the course of the inflammatory reaction, since these polypeptides exert potent vascular effects (vasodilation, endothelial cell separation) and are also capable of producing pain. Melmon & Cline (57) reported that human PMN, but not lymphocytes, generated pharmacologically active kinin from plasma or from purified α2-globulin substrate. The activity of human granulocytes was maximal at pH 7.2–7.6. Subcellular localization of the responsible protease was not established, although there was some evidence for an extra-lysosomal distribution. Kinin generation required intact cells capable of carrying on glycolysis and exhibiting active protein and RNA synthesis. A neutral, kinin-degrading enzyme was also detected in human PMN, but its action was independent of leukocyte metabolism and it could be recovered from disrupted cells.

An extensive series of studies on kinin production by leukocytes has been carried out by Greenbaum and his co-workers. They showed (58) that rabbit PMN released kinin activity from a bradykininogen substrate or from human plasma at acid pH. Kinin-forming activity appeared to be distributed in both lysosomal and extra-lysosomal fractions (12). Kinin-forming activity was also reported in alveolar and peritoneal macrophages of rabbits and in malignant leukocytes of mice (59) as well as in human peripheral lymphocytes (60). Greenbaum pointed out that rabbit leukocyte kinin-forming enzymes are not true kallikreins since they act at acid pH while kallikreins from other sources (e.g., human PMN) act optimally in the neutral range, and also because the PMN enzymes he has studied are not inhibited by the kallikrein inhibitor, aprotinin (Trasylol). In addition, he noted that the kinin produced by rabbit and mouse leukocytes is not bradykinin or its analogues, but a higher molecular weight polypeptide containing 24–25 amino acids (59). In parallel studies on kinin-destroying enzymes of white cells (61), this type of activity was detected principally in a rapidly-sedimenting (nuclear) fraction of the homogenate and was shown to be inhibited by aprotinin and to be maximally active at neutral pH.

Complement.—Chemotaxis of PMN is an integral component of immunologic and nonspecific inflammatory reactions. Under certain circumstances, PMN chemotaxis may tend to become self-perpetuating, thus greatly exacerbating PMN-mediated injury of tissues. In this context, PMN proteases have recently been implicated in several reactions which result in the cleavage of fragments from complement factors (notably C3 and C5), which fragments possess chemotactic activity for other PMN. Taubman et al (62) reported that a neutral protease of human PMN generated a chemotactically active fragment from purified human C3. The protease involved in this reaction did not appear to be the human PMN elastase described earlier, since the C3-cleaving enzyme was not active against synthetic elastase substrates.[2] A second neutral protease has been detected in rabbit PMN which generates chemotactic activity from C5 (63). This enzyme appears to be of larger molecular weight than the human PMN elastase, although it resembles the latter enzyme in that it is partly inactivated by soybean trypsin inhibitor.

MECHANISMS OF RELEASE OF PMN LYSOSOMAL CONSTITUENTS

The processes leading to extracellular release of lysosomal constituents from PMN are receiving increasing attention, in view of the growing evidence that these agents mediate a large part of PMN-dependent tissue injury. Two general mechanisms have been proposed to account for enzyme release. In the first, enzymes (and other granule constituents) are thought to be discharged upon the death of the cells, coincident with the loss of membrane integrity and bulk leakage of all cell contents. According to the second view, lysosomal components can also be released from viable leukocytes engaged in the act of phagocytosis. This mode of discharge can result from leakage out of digestive vacuoles if lysosomal contents have been secreted into the vacuole prior to complete closure of the vacuole's opening ("regurgitation"). Alternatively, leakage may occur by virtue of direct extracellular discharge of lysosomal granules through fusion of lysosomal and plasma membranes ("reverse endocytosis"). The latter could result, in turn, from molecular alterations within the involved membranes triggered by the phagocytic act.

In a recent experiment (64), it was shown that immune complexes distributed along a nonphagocytizable surface (such as a micropore filter) will stimulate lysosomal discharge from PMN to the exterior of the cell. Under these circumstances, considerably less of the immune complex is required to stimulate discharge of granule constituents than is required to induce comparable discharge during phagocytic uptake of the same immune reactants in suspension. This in vitro model may well have relevance to modes of enzyme release in vivo when PMN are attracted to immune complexes deposited along basement membranes of glomerular capillaries or along the internal elastic lamina of arterial vessels.

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[2] Taubman, personal communication.

The classical view of tissue breakdown by relatively short-lived PMN held that enzymes were principally released upon the death of the cells (1). This mode of release appears to be the likeliest following encounters between PMN and cytotoxic monosodium urate crystals in gout (65), or between PMN and bacteria which elaborate leukocidal toxins (certain pyogenic microorganisms). On the other hand, a number of studies (for a recent review, see ref. 66) have provided evidence of lysosomal leakage from viable PMN through "regurgitation" or "reverse endocytosis" during phagocytosis of inert particulates or of immune reactants in vitro. The release of lysosomal hydrolases following phagocytosis of immune reactants (e.g., complexes of heat-aggregated IgG and rheumatoid factor) appears to be modified by intracellular levels of cyclic AMP. Thus, prostaglandin E_1 (an agent which activates adenyl cyclase, thereby increasing cyclic AMP levels) and theophylline (an inhibitor of the cyclic AMP-destroying phosphodiesterase) have been shown to act synergistically to suppress leakage of lysosome contents from phagocytizing PMN. The cytoplasmic microtubule system (which is also involved in other cell processes modified by cyclic nucleotides) may represent the site of action of cyclic AMP in regulating lysosomal leakage, by virtue of guiding intracellular movements of lysosomes during phagocytosis (66, 67).

Future investigation will be required to determine whether immunologically induced, PMN-mediated tissue injury in vivo (such as the Arthus reaction) can also be ameliorated by drugs which raise cyclic AMP levels in leukocytes and thereby reduce their discharge of lysosomal hydrolases. It should be noted that cell death and bulk release of enzymes may supervene in vivo not many hours after the initial encounter of PMN with immune reactants deposited in vessel walls, just as it does (albeit earlier) in pyogenic infections and gout.

ENDOGENOUS INHIBITORS OF PMN PROTEASES

SERUM ANTIPROTEASES

Even the earliest biochemical studies of tissue damage by inflammatory cells stressed the critical balance between leukocyte enzymes and tissue antienzymes in determining the degree of injury produced (28). In the intervening years, much new information concerning the nature and mechanisms of action of endogenous antiproteases has been gained. This subject has been reviewed recently (18, 68).

One of the major endogenous serum inhibitors influencing PMN neutral proteases is the alpha_1 antitrypsin (A_1AT), a glycoprotein of approximately

60,000 molecular weight present in normal serum at 200–500 mg percent concentration and responsible for 90 percent of the trypsin-inhibiting capacity of the serum (normally about 1–2 mg trypsin/ml serum). As mentioned earlier, the capacity of this inhibitor to complex with and inactivate trypsin is sharply curtailed below pH 5.0, and its effectiveness against PMN proteases may be compromised by PMN-produced lactic acid in inflammatory exudates.

Evidence for inhibition of the spontaneous fibrinolytic activity of human PMN at neutral pH by the A_1AT fraction of human serum was obtained by Keuppers & Bearn (19). More recently, we have shown that A_1AT and, to a lesser extent, the α2-macroglobulin inhibitor of serum, also inhibits the neutral elastase activity of human granulocyte lysosomes. This conclusion was based on experiments using A_1AT-deficient sera in an elastoproteolytic assay system, and on tests with purified human A_1AT and α2-macroglobulin in an elastoesterolytic assay (46).

A heritable disorder leading to A_1AT deficiency states (69) may be associated with exacerbation of tissue injury by PMN neutral proteases. Among the susceptible targets for PMN enzymes in this condition may be lung elastin and other alveolar wall proteins, as well as glomerular basement membrane and arterial elastica (70). The role of proteases (especially those of PMN and alveolar macrophages) in the pathogeneis of pulmonary emphysema associated with A_1AT deficiency is currently receiving increased attention.

PROTEASE INHIBITORS WITHIN PMN

Davies & Weissmann (31) and Davies et al (32) identified an inhibitor in the cytosol fraction of rabbit PMN which suppressed the activity of a neutral, histone-degrading protease present in the granule fraction of those cells. In their experiments, histonase activity of whole cell lysates was considerably lower than that of isolated granules, rendering it unlikely that lysed PMN in rabbit tissues would inflict proteolytic damage via the histone-degrading enzyme. On the other hand, alternative mechanisms for lysosomal enzyme release without accompanying cell lysis have already been discussed (see above). The present author (71) has reported that human PMN cytosol can similarly inhibit the elastase present in human PMN granules (although the degree of inhibition in whole cell lysates is much less than that observed in the rabbit histonase-antihistonase system). Inhibition of PMN elastase appeared to be selective for the lysosomal enzyme, in that pancreatic esteroproteases (including pancreatic elastase) were not affected. On the other hand, other human PMN granule hydrolases (acid-*p*-nitrophenylphosphatase and beta-glucuronidase) were also slightly inhibited by PMN cytosol fractions.[3] Further studies have ruled out RNA and other nonspecific polyanions of cytosol as being responsible for elastase inhibition, and suggest instead that the inhibitor activity resides in a fraction of cytosol containing large molecular weight, acidic. heat-stable proteins, immunologically distinct from A_1AT and α-2-macroglobulin inhibitors of serum (71, 72). One possible role for cytoplasmic inhibitors of the type described above may be the protection of cells against slow intracellular leakage of lysosomal hydrolases active at neutral pH. It seems less likely that such inhibitors would interfere significantly with hydrolytic reactions involving rapid, bulk release of lysosomal enzymes, for example, in autolysing PMN. (Sterile human pus and homogenized human PMN retain considerable proteolytic activity at neutral pH.) Neither would cytosol inhibitors be expected to interfere with the action of enzymes released from viable phagocytizing leukocytes, where soluble cytoplasmic components are retained.

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[3] Janoff and Blondin, unpublished observations.

PMN PROTEASES AND COCARCINOGENESIS: A SPECULATION

In addition to degrading interstitial components of connective tissues, PMN proteases may also alter the surface properties of living cells in inflamed tissues. If true, this may have significance for the process of carcinogenesis, since exposure of cultured mouse fibroblasts to nonlethal concentrations of trypsin or plant proteases such as papain and ficin has been shown to result in a transient alteration in growth behavior of the affected cells occurring simultaneously with the exposure of normally masked chemical groupings on the cell surface. The latter can be detected by virtue of enhanced agglutination of the cells by a variety of plant lectins (73–76). The alteration in cell growth behavior which occurs under these conditions is highly reminiscent of the loss of contact inhibition observed in virally transformed fibroblasts, except that growth stimulation induced by brief treatment with proteolytic enzymes is transient in nature and normal growth control reappears as soon as the cells are able to restore their original surface structure.

An interesting possibility is that chronic or intermittent irritation of tissues may lead to repeated exposure of cells to proteases released from leukocytes with a consequent long-term effect upon cellular growth habit in the irritated tissues. The classic hyperplasia of epithelial linings observed in response to chronic irritation may be based, in part, on such a mechanism. At the same time, the hyperplastic response to cocarcinogens appears to be intimately associated with their tumor-promoting action. In this context, it is worth noting that protease inhibitors have recently been shown to suppress tumor promotion by irritant cocarcinogens (77, 78).

PMN proteases have not yet been tested for their ability to alter growth behavior or agglutinability of cultured cells in a fashion similar to that which attends treatment with trypsin, ficin, or papain. However, in view of the fact that PMN of most species contain both acid and neutral proteolytic enzymes with sufficiently comparable properties to those listed above, an examination of this possibility may well be worthwhile.

CONCLUSION

By virtue of their phagocytic and intracellular killing activities, PMN serve a defensive function in tissues invaded by infectious organisms. They are also rapidly marshalled at tissue sites exposed to noninfectious but nevertheless foreign and potentially noxious substances. Removal and degradation of antigens complexed with host antibody is therefore another frequent activity of these cells. PMN substances involved in intracellular killing and degradation are sometimes released extracellularly as well. When this occurs to a degree sufficient to overwhelm host inhibitors (e.g., antiproteases), the degree of tissue damage produced by PMN may significantly diminish their beneficial effects. Thus, in some acute inflammatory diseases of immunological origin, the action of PMN may be largely destructive to the host, and control of PMN-mediated tissue damage is desirable. In order to devise rational means of exercising such control, knowledge of the specific PMN agents capable of causing tissue damage is helpful. Towards this end, we have attempted to review some of the more recent information on PMN proteases involved in tissue injury. Hopefully, further progress in this subject will prove useful in the search for improved means of controlling inflammatory disease.

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INTERSPECIES PATTERNS OF SLOW VIRUS DISEASES

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A concise definition of slow virus diseases is difficult to achieve, as criteria for this group of diseases are unclear. It is likely that several types of agents are incorrectly considered as slow viruses at present. As more is learned about them there will probably be considerable reclassification of the diseases so designated.

The term "slow infection" is not new. In the early 1950's Sigurdsson (1–3), an Icelandic pathologist, applied the name to maedi, a slowly progressive pneumonia of sheep, to rida, a chronic encephalitis of sheep (probably identical to scrapie), and to Johne's disease, a mycobacterial infection of the intestine of cattle. Sigurdsson's criteria were: (*a*) a long latent period lasting from several months to several years; (*b*) a protracted course, usually ending in death; and (*c*) strict host specificity and sometimes familial predisposition for the specific disease.

Other characteristics now known to be associated with slow viral infections are persistence of the agent in the infected host for much or all of its life, and a long, continued host response which may in itself be part of the disease process (e.g., circulating antigen-antibody complexes). In general, however, the host responses are minimal. Although it was some time before Sigurdsson's ideas became widely accepted, the concept of slow infections has exerted considerable influence on thinking about diseases of a progressive, degenerative nature with long course and obscure etiology. Interpretation of Sigurdsson's theory by comparative pathologists has opened an entirely new field of research in chronic diseases. This work has recently been reviewed by Abinanti (4), Field (5), and Gibbs & Gajdusek (6).

It has been said that there are no slow viruses, only slow virologists. This statement serves to illustrate the difficulty of understanding these important but mysterious diseases. The most perplexing aspect has been the time interval of one, five, or even twenty years between initial exposure and entry of the infectious agent into the host, and eventual overt expression of the disease. Thus, even though it may be improper to refer to these as slow viruses, for some of them replicate rapidly, the delay in expression of the diseases has been the chief obstacle to understanding their pathogenesis.

In retrospect, this possibility should have been anticipated, for extended incubation periods, delay of onset, and persistence had long been recognized in other viral diseases such as rabies and herpes simplex. More recently, rubella and cytomegalovirus have been disclosed as vertically transmitted persistent viral diseases. However, attention has been focused on such viral diseases as poliomyelitis, influenza, equine encephalitis, and others with a short incubation period and acute course, usually resulting in complete recovery of the host with solid immunity, or in death. Antibody production is usually swift and specific. Study of the latter diseases has been relatively simple because the antibody response has made detection straightforward.

SCRAPIE

Scrapie has been known as a disease of sheep for nearly 200 years in England, Scotland, and continental Europe, but its manner of transmission has remained elusive. Some investigators felt that it was a genetic disease, whereas others believed it to be transmitted by infection in utero or at coitus (7). The name of the disease is descriptive of one clinical form in which the affected animals rub against fixed objects, resulting in patchy loss of wool. In France the disease is known as "la tremblante," which describes the trembling syndrome often associated with it. Investigations over the past fifty years have shown that the agent is transmissible and that it has many characteristics of a virus. However, certain features of its epizootiology, pathologic manifestations, and biochemical and physical characteristics are unusual. These include the very long latent period, and its resistance to heating and chemicals that inactivate most viruses (8–11).

The peculiar nature of scrapie was of interest mainly to veterinarians and sheep ranchers until 1959, when Hadlow (12) observed a close resemblance of the pathological lesions and clinical signs of sheep scrapie to kuru in man. This was confirmed by Beck (13). This stimulated intensive re-examination of the human disease and led to the suspicion that kuru and other progressive central nervous system diseases of man may be caused by "slow" viruses. The lack of easily manipulable quantitative systems to study these agents has produced some inconsistent data and many inconclusive experiments. It was reported in 1964 by Pattison & Sansom (14) that the scrapie agent was partially dialyzable through a membrane with a pore diameter of 2.4 millimicrons. On the other hand, it was subsequently found by gradocol membrane studies to have a size between 27 and 43 millimicrons (Gibbs & Gajdusek, (15); Gibbs, (16). The accumulation of quantitative information has been slow and difficult. Earlier observations were made after inoculating sheep and goats and subsequently recording their clinical reactions and pathologic lesions, but results became more precise after 1961 when Chandler (17) successfully adapted the agent to mice with reduction of incubation period. However, even this means of quantitation depends heavily on the judgement of the observer conducting the experiment. This caution must be kept in mind whenever one assesses descriptions of the behavior of the agent, such as its great resistance to formalin (18) and ionizing radiation (19), its negligible loss of titer following high doses of ultraviolet light (20), and its resistance to boiling (21).

The lesions of scrapie are not striking; in fact they are characterized by a paucity of tissue changes which are confined to the brain and spinal cord (13). These consist of enlargement and proliferation of astrocytes, the formation of vacuoles within the cytoplasm of nerve cells (22), and vacuolation or pooling of unidentified substance in intercellular spaces of the brain. Extensive immunological studies have failed to identify any specific reactions. Ultrastructural studies have demonstrated myelin figures in microglia, laminated and pleomorphic inclusion bodies in neurons, and interstitial accumulation of amyloid bodies. These observations have been of limited help in learning more about the scrapie agent (23–25).

KURU

In 1959, Hadlow (12) called attention to the similarity between the lesions seen in scrapie of sheep and in kuru, a progressive, degenerative encephalopathy of natives of the Fore tribe in New Guinea, originally described by Gajdusek & Zigas (26). Extensive trials have since confirmed that kuru is indeed an infectious disease and that it has many clinical and pathologic similarities to scrapie. The disease was reproduced by inoculation of filtered material into chimpanzees and was subsequently transmitted by serial passage to additional chimpanzees, and spider, squirrel, and capuchin monkeys (27–30). It should be emphasized that there is no evidence that kuru and scrapie are caused by the same agent; the agents merely have similar characteristics but affect different species.

The pioneering studies of Gajdusek and his colleagues have brought kuru to the attention of the entire medical world and their work with the natives of New Guinea has virtually eliminated this disease, which was formerly the commonest cause of death in the Fore tribe. This has resulted in extension of these investigations to other diseases which are less restricted in their demographic distribution.

TRANSMISSIBLE MINK ENCEPHALOPATHY (TME)

In 1965, a disease of mink which resembled scrapie was reported by Hartsough & Burger (31) in Wisconsin. It had been observed sporadically since 1947 but was not previously described in the literature. Subsequent studies have shown it to be a filterable, transmissible agent which is very resistant to formalin. The lesions produced in the mink are remarkably similar to those of scrapie in sheep (32–34).

The agent has been transmitted to the Rhesus monkey and the squirrel monkey (35, 36), but attempts to infect mice, cats, calves, and chickens have thus far been unsuccessful. At this time the relationship between the agents of transmissible mink encephalopathy and scrapie is still unsettled, but the TME agent constitutes another of the elusive, slow-reacting filterable agents which can neither be seen nor clearly identified except by their effect on the host.

There are important analogies between scrapie, kuru, and mink encephalopathy. The peculiar host ranges of these diseases as illustrated in Table 1 and their similarity to other supposedly noninfectious diseases, coupled with the great difficulty encountered in proving their infectious nature, make it seem inevitable that additional examples will be disclosed in man and other animals.

Multiple Sclerosis (MS)

Multiple sclerosis is one of the most mysterious degenerative diseases of the central nervous system. Poskanzer (37) has pointed out that the epidemiologic patterns exhibited by MS resemble those of a common subclinical infectious disease with occasional overt manifestations and an incubation period of about twenty-one years. Transmission attempts in animals, however, have been unsuccessful, and the search for an infectious agent has thus far been fruitless. In one study, Palsson et al (38) injected brain material from a patient affected by multiple sclerosis into sheep, and scrapie-like symptoms and lesions developed in these animals but not in controls given normal human brain inoculum. This has not been subsequently confirmed, but the evidence in the one experiment was strong.

Even though the evidence for the infectious nature of multiple sclerosis and similar diseases of man is difficult to substantiate, indications from experiments with kuru and the one report of the possible transmission of multiple sclerosis to sheep have stimulated a great amount of research on several other diseases.

Subacute Sclerosing Panencephalitis (SSPE)

This condition, also known as Dawson's encephalitis (39), is a progressive neurologic disease of children characterized by intracytoplasmic and intranuclear inclusions in the neurons and glial cells. These inclusions are composed of tubular forms which resemble nucleocapsids of viruses of the paramyxovirus group (40). Additional evidence for the infectious nature of

TABLE 1. Host Range of Certain Slow Virus Diseases

Disease	Primary host	Adaptable to
Kuru	Man	Chimpanzee, spider monkey, squirrel monkey, capuchin monkey
Scrapie	Sheep	Goat, mouse, hamster rat
Mink encephalopathy	Mink	Rhesus monkey, squirrel monkey

this disease has been reported from cell culture studies with African green monkey kidney cells. In addition to paramyxovirus structures, there appears to be growth of another virus, perhaps of the papova group. Koprowski and co-workers (41) have expressed the hypothesis that an interaction between myxovirus and papovavirus within the cells may be responsible for the production of this disease. Horta-Barbosa et al (42) reported isolation of an agent from a patient by co-cultivation of brain ells with HeLa Cells, and Byington et al (43), employing alternate passages in cell cultures and in two-day-old hamsters, reported adaptation and reisolation of the agent. Patients with clinical evidence of this disease and with nucleocapsid antigen within the brain cells develop high antibody titers against measles virus (40). An uncommon disease of dogs known as "old dog encephalitis," in which intranuclear inclusion bodies are found in the neurons, may be the closest counterpart in other animals to SSPE.

Spongiform Encephalopathy

The evidence for viral etiology is also strong in spongiform encephalopathy (Creutzfeldt-Jakob disease), a term applied to a group of subacute, presenile encephalopathies of man characterized by dementia, involuntary movements, and in certain cases by severe spongiosis of cortical grey matter accompanied by loss of neurons and astrocyte proliferation. Brain material from one such case was inoculated intracerebrally into a chimpanzee by Gibbs et al (44). Thirteen months later the animal developed subacute, progressive degenerative brain disease almost identical to that of the human patient (45).

Progressive Multifocal Leukoencephalopathy (PML)

This is a subacute human neurological disease first described in 1958 by Astrom, Mancall & Richardson (46). Since then there have been nearly 100 cases reported, many of them associated with Hodgkin's disease or chronic lymphocytic leukemia, and originally PML was regarded as a late complication of one of the immunoproliferative diseases. Reports by Zu Rhein & Chou (47), Zu Rhein (48, 49), and Muller & Watanabe (50) indicate that PML may be a latent infection with a virus of the papova group, which by some unknown mechanism becomes activated and manifests itself by attacking the central nervous tissues. The significance of the disease, when viewed in light of recent disclosures about kuru, scrapie, SSPE, and other diseases, is that a yet undisclosed infectious disease of the central nervous system or other systems may be caused by "latent" or "temperate" viral infections. It is true that no viral agent has been isolated from PML, but by skillful use of the electron microscope and correlation of pathological to clinical data a strong association has been shown between the presence of virions and expression of the disease.

ALEUTIAN DISEASE (AD)

Aleutian disease is an infection of mink so named because it originally was thought to affect only the color phase of mink known as Aleutian. It is now known that all mink can contract this disease, although there appears to be some predilection for more serious disease in those animals possessing the Aleutian genotype (51–54).

Outbreaks of this disease occurred on mink ranches in the United States following the injection of formalinized vaccines prepared for use against other viral diseases. Work with AD has been hampered by the inability to grow AD virus in cell cultures. Preliminary findings in our laboratory indicate that cultures established from mink with AD cultured in the presence of ^{3}H uridine secrete labeled material into the culture medium that bands in an isopycnic sucrose density gradient at a density of 1.20–1.22. Such a peak is not found when the cells are labeled with ^{3}H thymidine rather than ^{3}H uridine. The labeled peak is RNase-resistant but appears to be RNA by other criteria.

Aleutian disease replicates rapidly in both Aleutian and non-Aleutian mink (55, 56). Quantitative in vivo growth curves of AD virus show maximal virus proliferation ten days after infection. Infected mink also exhibit a prompt antibody response to AD virus but the antibody does not appear to reduce the severity of the disease. The antibody titer was quantitated by an indirect fluorescent antibody technique described by Porter (56).

The glomerulonephritis regularly found in AD, accounting for the majority of deaths, is probably mediated by immune complexes (57–59). Elution studies of affected glomeruli indicate that the globulin in the glomeruli has some activity against AD virus, as demonstrated by Porter's indirect fluorescent antibody test. The eluates did not possess antinuclear activity, although antinuclear antibodies have been found in mink sera (60).

The biological significance of AD is that it constitutes a persistent viral disease in which, even though the host responds with specific antibodies, the agent continues to replicate. This results in damage to various organs by the depostion of complement and antigen antibody complexes. Also the question of its relationship to multiple myeloma is open because of the transition from an electrophoretically heterogeneous hypergammaglobulinemia to homogeneous myeloma-like hypergammaglobulinemia in certain mink with AD (57, 61, 62).

PROGRESSIVE PNEUMONIA, MAEDI, AND VISNA

Progressive pneumonia is a chronic pulmonary disease of Montana sheep that closely resembles maedi, a pulmonary disease of Icelandic sheep. The progressive pneumonia virus (PPV) is antigenically and morphologically similar to maedi and another closely related virus, visna, which causes a slow, demyelinating disease in sheep (63, 64). Serological studies on the antigenic relationships among a number of strains of maedi and visna indicated that the two viruses share common antigens but are distinct (65–67). Recent fluorescent antibody studies have established the antigenic relationship of PPV to visna and maedi (68).

Surface budding and free extracellular particles containing an electron-dense nucleoid have been observed in cell cultures infected with visna and PPV (68). These particles bear a striking resemblance to the oncogenic RNA viruses. Other similarities among visna and maedi viruses and RNA tumor viruses include size and structure, mode of maturation, requirement for cellular DNA synthesis, and nucleic acid composition. A recent fascinating finding is that visna and possibly PPV virus contain an RNA-dependent DNA polymerase (reverse transcriptase) (69, 70). This enzyme has been found in RNA-containing avian and murine leukemia-sarcoma viruses (71, 72) as well as in C-type RNA viruses of the cat, hamster, and viper (73). This polymerase is not generally associated with nononcogenic RNA viruses; therefore, its presence in the nononcogenic slow virus suggests a role in replication and pathogenesis. It is also noteworthy that all three of these slow virus diseases cause bronchiolar proliferation that may develop into true neoplasia. These two viruses also cause transformation of murine cells (74). Infectious virus could not be detected in the transformed cell lines, but was recovered from all cell lines when they were co-cultivated with normal sheep testis. The recovered virus was visna or PPV, which retained its capacity to transform mouse cells. Thus, by biologic and physicochemical criteria, the slow viruses appear similar to RNA tumor viruses, which could also be considered slow viruses. The possibility of antigenic similarities, such as the group-specific antigen (GS-3), should be explored.

Equine Infectious Anemia (EIA)

Equine infectious anemia is a viral disease of the genus *Equus* (75). It is arthropod-borne and is therefore seasonal, occurring most commonly in swampy areas. There have been reports of transmission by contact, but these have not been confirmed (76). Some of the peculiarities of EIA are so fascinating that the disease must be considered in context with comparative aspects of the entire spectrum of the slow virus diseases. It is usually chronic, with periods of exacerbation often following stress. There is persistent viremia, with some animals having been known to carry infective virus in their blood for as long as eighteen years. As in Aleutian disease, there have been reports of transmission to man, but these have not been adequately substantiated (77). Attempts to infect other animals such as calves, lambs, swine, and guinea pigs have been unsuccessful (78). The agent has been propagated in leukocyte cultures (79, 80), and a virus has been observed in infected leukocyte cultures by electron microscopy (81). These are important findings which may enable quantitative in vitro studies of one persistent virus disease.

Immune processes appear to be active in the pathogenesis of EIA. Hypergammaglobulinemia and hemolytic anemia are consistently present

(82). There is infiltration of liver and lymphoid tissue by plasma cells, and periarteritis and glomerulonephritis are occasionally present. Thus, it resembles Aleutian disease of mink in several ways. The erythrocytes of infected horses are coated with C3 fraction of complement (83), and globulin and C3 complement are deposited in the renal glomeruli (84). Plasma C3 levels are also reduced in infected animals (85).

LACTIC DEHYDROGENASE (LDH) VIRAL INFECTION

Lactic dehydrogenase virus is a small RNA virus. The natural host is the mouse, and infection results in a 5- to 10-fold increase in the activity of plasma lactic dehydrogenase, as well as other circulating enzymes, due to impaired clearance of endogenous enzyme (86). Lactic dehydrogenase virus infection also affects the functional capacity of the immune system (87). Antibody producion is enhanced while tolerance, cellular immunity, and phagocytosis are depressed. The impaired plasma enzyme clearance and derangement of immunological function may be due to replication of the virus in cells of the reticuloendothelial system.

The virus persists in the circulation in the form of an infectious virus-antibody complex, and deposits of these complexes in the kidneys produce a mild immune-complex type of glomerulonephritis (87).

LYMPHOCYTIC CHORIOMENINGITIS (LCM) VIRAL INFECTIONS

LCM virus is another small RNA virus. The true host is the mouse but infection can occur in man and variety of other animals. Circulating virus-antibody complexes lodge in the kidney (88). The glomerulonephritis produced is severe in contrast to the mild immune-complex type of glomerulonephritis associated with LDH virus infection. Other lesions, in several murine strains persistently infected with LCM virus, are focal hepatic necrosis and interstitial lymphoid infiltration in many organs. The pathogenesis of the nonglomerular tissue injury is in part related to the interaction of anti-LCM antibody with viral antigen at the tissue site (88).

The major characteristics of chronic LDH and LCM viral infections may be found in other slow virus diseases. Thus, in Aleutian disease, equine infectious anemia and possibly other chronic viral infections, viremia, circulating virus-antibody complexes, and deposition of these complexes in the glomeruli occur.

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PULMONARY INVOLVEMENT IN HYPOVOLEMIC SHOCK[1]

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INTRODUCTION

In the last decade, there has been a remarkable increase in interest in pulmonary problems in critically ill patients. This interest has been stimulated by two separate sources, the intensive care units in civilian hospitals in this country and the armed forces hospitals in Indochina. Both have come to the conclusion that the most prominent problem in the management of critically ill patients is often progressive, unexplained pulmonary insufficiency in patients without previous pulmonary disease (1).

It is very likely that the emergence of pulmonary insufficiency as a major problem in critically ill patients is the result of our heightened ability to deal successfully with problems that previously were lethal. For example, the successful management of hemorrhagic hypotension depended on development of the science of blood banking, and the successful management of renal problems after injury depended on the development of methods of prevention and on renal dialysis techniques. Equally as dramatic has been the utilization of our techniques for treatment of acute trauma in Vietnam. Every combat soldier is no more than 60 minutes from an air conditioned, fully equipped and staffed hospital possessing the most sophisticated instrumentation plus enormous supplies of whole blood. Casualties with major injuries who would have died in previous conflicts are now reaching these hospitals alive. It is in these major trauma victims that pulmonary insufficiency may develop.

Critically ill patients, whether in a civilian intensive care unit or military hospital in Vietnam present highly complex problems in management. Most have suffered a period of hypovolemic hypotension; many are septic as well as hypovolemic; fluid therapy often includes large volumes of blood and asanguinous fluids; intubation and ventilatory support are frequently needed; and various concentrations of O_2 in the inspired air are administered. In this setting, it is difficult to sift out the various factors that might be responsible for a deterioration in lung function.

In this chapter we shall focus our attention on a few key issues relating to the lungs, including the physiologic effects of hemorrhage, the morphologic effects of hemorrhage, and the effects of treatment.

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THE PHYSIOLOGIC EFFECTS OF HEMORRHAGE

O_2 transport.—One of the fundamental issues concerning the relationship between hemorrhagic hypotension and respiratory function is the question of oxygen transport. Current information suggests that in the pure hemorrhage setting, O_2 transport does not appear to be grossly impaired, at least during several hours of hypotension (2). Cournand (3), in 1943, showed that arterial O_2 saturation in man were unaffected by hemorrhage. In our laboratory, we have shown that pure hemorrhage in the baboon is associated with a significant elevation in arterial pO_2 while the animal is breathing room air. This elevation is likely the result of an increase in minute volume as evidenced by a significant fall in pCO_2 in response to the development of metabolic acidosis usually seen with hemorrhagic hypotension. If these same animals are then exposed to 100 percent O_2 concentrations in the inspired air for 20 min, the pO_2 rises to approximately 500 mm/Hg, the same value observed in the control state (4). This response of O_2 breathing indicates pulmonary A-V shunting of less than 10 percent, a normal respiratory response.

Dead space tidal volume ratios.—The effect of hemorrhage on dead space ventilation has been studied by several investigators. Theoretically, hemorrhagic hypotension should be associated with an increase in the dead space tidal volume ratio (Vd/Vt), since, as pulmonary artery pressure and flow fall, areas of the lung remain ventilated but are not well perfused. These underperfused areas functionally resemble the anatomic dead space in that they no longer participate in gas exchange. During hemorrhagic shock in the dog, Gerst et al (5) showed that the Vd/Vt increased from .33 to .55. These dogs were on controlled ventilation. In spontaneously breathing baboons, we were unable to show a significant increase in Vd/Vt following hemorrhage (2). However, it should be noted that in spontaneously breathing animals, the interpretation of sequential measurement of Vd/Vt becomes complicated since a significant negative linear relationship exists between Vt as measured with a gas meter and Vd/Vt as measured by CO_2 kinetics. Thus, in a spontaneously breathing individual, a rising Vd/Vt may indicate either an increase in dead space, or simply a decrease in the tidal volume. The sequential measurement of Vd/Vt is therefore most useful in a setting where the respiratory rate and tidal volume are held constant. If respiration is spontaneous, tidal volume measurement must be obtained along with Vd/Vt ratios, in order to interpret the results accurately. On the basis of Gerst's studies and others, it is reasonable to assume that hemorrhagic hypotension does produce an increase in dead space and therefore tidal volume may have to be adjusted upward to maintain normal CO_2 excretion.

Lung compliance.—Lung compliance is another index of lung function that has been examined following a period of hemorrhagic shock. It is defined on the ratio of the change in tidal volume divided by the change in air way pressure ($\Delta V/\Delta P$). One might expect that if the lung is damaged during the shock period, it would be reflected in a fall in compliance. In the study reported by Gerst et al (5) in dogs, tidal volume was held constant, so that changes in air way pressure reflected changes in compliance. They found a small fall in compliance (90 percent of control) when 42 percent of the blood volume was removed. Buckberg and his associates (6) found no changes at all in compliance following hemorrhagic shock in the baboon. These baboons were bled into profound hemorrhagic hypotension for two hours and in some animals the lungs were then excised. Pressure volume curves were generated in a specially designed plastic box. No differences in lung compliance could be found between the shocked and nonshocked animals. In our laboratory Proctor et al (4) came to a different conclusion from either of the above two authors. A series of baboons were subjected to profound hemorrhagic shock for two hours. During this interval, tidal volume was measured with a pneumotachygraph and transpulmonary pressure was measured as the difference in pressure from the endotracheal tube and esophageal balloon. It was found that rather than declining, compliance rose significantly from a baseline value of 128 ml/cm H_2O to $160 \pm .07$ at the end of the hemorrhage period.

Each of the three investigators has a different view regarding the effects of pure hemorrhage on the lungs. Gerst believes that progressive atelectasis is the principal factor contributing to falling compliances in the dog. Buckberg suggests that hemorrhage does not produce a serious lesion in the lung. Proctor states that the alteration in compliances noted in his studies reflect the fall in pulmonary blood volume. Gerst's position is supported by histological data in the dog showing diffuse atelectasis following hemorrhage. However, the histologic changes noted in the dog are not seen in the baboon. Measurements of central blood volume in the dog and baboon following hemorrhagic shock confirms Proctor's idea that a major reduction in central blood volume does occur secondary to hemorrhage.

Salzano and his group (7) have pointed out that modest blood loss appears to be associated with a fall in compliance as noted in Gerst's report while massive hemorrhage appears to be related to an increase in compliance as in Proctor's investigations. The explanation for these changes remains unclear. It does seem safe to conclude that a severe stiffening of the lung is not generally found in association with profound hemorrhagic shock in the primate. In fact, the pulmonary system in general seems to function reasonably well during pure hemorrhagic hypotension.

The effects of nonthoracic trauma plus hemorrhage.—In contrast to the effective response of the respiratory system to pure hemorrhagic shock, the combination of hemorrhage plus nonthoracic trauma produces quite a different picture. Several investigators have reported a surprisingly high incidence of hypoxia in Vietnam combat casualties with severe injuries not involving the thorax. The first reported use of blood gas measurements in a combat area was by Collins et al (8) in 1968 in an area just north of Saigon. They studied 68 patients with severe extremity injuries. Fourteen of these patients demonstrated admission pO_2 levels below 80 mm/Hg. All 68 patients had normal pCO_2 values, indicating normal minute ventilation. No correlation with hypoxia could be found with any of the following variables: administration of morphine, amount of blood transfusions, interval from injury to treatment, or level of serum lactate. A relationship was found between the site of injury and hypoxia. Six of thirteen patients with high velocity injuries to the femur without blast amputation were hypoxic on admission. Although no firm conclusions could be drawn regarding the etiology of this hypoxia, Collins and his associates suggested that fat emboli originating from the injured limb, were the most likely factor.

A similar study was reported the following year from the DaNang area by Lowery et al (9). Sixty-six badly injured combat casualties were studied sequentially for four days. The authors reported normal pO_2 levels on admission but then noted a drop in the 12-hr point to 70 mm/Hg. They found no correlation of the hypoxia with morphine administration, amount of blood transfused, or asanguinous fluids infused. A relationship of hypoxia to blast amputations was noted, in contrast to the earlier reports by Collins et al of hypoxia with femur injuries but without blast amputations. In addition, almost all patients in the DaNang study showed biochemical evidence of fat emboli whether or not they developed pulmonary insufficiency. Thus, the etiology of the hypoxia remains unclear, especially concerning the role of fat emboli.

The results of these two studies indicate that a major degree of hypoxia can be expected in a large percentage of patients suffering from a combination of trauma plus hemorrhage. Information regarding the pattern of ventilation in this setting in man with nonthoracic trauma was reported in 1970 by Proctor, Ballantine & Broussard (10), again from the DaNang area. They studied 15 control patients, 33 massively injured patients and 4 patients with serious post-injury pulmonary insufficiency. Respiratory mechanisms were studied every 6 hr for three days by obtaining tidal volume measurements from a pneumotachygraph and transpulmonary pressure recordings from a esophageal balloon and endotracheal tube. These primary signals were fed on line into a specially designed analogue computer, which calculated a number of indices of respiratory mechanics. Massive injury was associated with a reduction in tidal volume from a normal of approximately 600 cc to a low value of 413 cc 12 hr after admission. At the same time, respiratory rate rose from 16 to 21. Minute volume was therefore not radically altered. Pulmonary arteriovenous shunting rose from 11 percent to a high of 30 percent. Compliance was unchanged from normal. The results of the study emphasize two important points. First, that the hypoxia associated with hemorrhage plus trauma is related to intra-ultrapulmonary shunting.

Second, the pattern of ventilation is altered in that tidal volume is depressed and respiratory rate increased. This ventilation pattern is dangerous since it has been shown that rapid shallow breathing favors diffuse alveolar collapse. Such patients often require ventilatory support in order to increase tidal volume levels. These data should encourage early and repeated measurements of tidal volume as well as blood gases.

MORPHOLOGIC CHANGES IN THE LUNG ASSOCIATED WITH HEMORRHAGIC SHOCK

Until very recently, pulmonary morphologic studies following hemorrhagic shock were limited, in large measure, to light microscopic studies in dog models. The dog is a poor model for these studies, since the canine pulmonary response to hemorrhage is quite different from the primate's. Henry and his associates (11) have shown that during hemorrhagic shock in the dog, pulmonary artery pressure often rises above control values, while left atrial pressures remain low. Following retransfusion, pulmonary artery pressures frequently climb even higher. These results suggest that an extreme elevation in pulmonary vascular resistance develops during shock and resuscitation. In the primate, in contrast, pulmonary artery pressure falls during hemorrhagic hypotension and returns to baseline following retransfusion. The canine pulmonary vascular system therefore appears to develop a congestive pattern similar to that seen in the splanchnic circulation of the dog following hemorrhagic shock. Indeed, morphologic studies in the dog following hemorrhagic shock have revealed diffuse congestive atelectasis (12).

We have recently completed a series of light and electron microscopic studies of the baboon lung following 2 hr of hemorrhagic shock (13). In these studies, the lungs were fixed in vivo by simultaneous dual perfusion through the pulmonary vascular and endobronchial systems. Special light microscopy techniques were employed involving sections of Epon-embedded tissues 0.5 to 2μ thick.

By light microscopy, multiple areas of the lung were viewed. Sections from normal lung tissue and from lungs following 2 hr of hemorrhagic shock are shown in Figures 1a through 1d. Diffuse interstitial edema of the thick portion of the alveolar capillary membrane was the principal post-shock finding. These changes were investigated in more detail by electron microscopy of glutaraldehyde-fixed lung tissue. In Figures 2a and 2b, the normal anatomy of the lung is shown. Note the close approximation and periodicity of the collagen fibers in the alveolar capillary membrane. Also note the presence of pinocytosis, especially in the endothelial layer.

The effect of 2 hr of hemorrhagic shock is shown in Figures 3a through 3d. Figure 3a demonstrates a low power view of the alveolar capillary membrane. A substantial amount of interstitial edema has appeared when compared to the control state. Large electron-lucent spaces in the interstitium are evident and dispersion of individual collagen fibers can be seen. The endothelial and epithelial layers appear intact. In most sections viewed, the capillary spaces are devoid of cells. Where cells are present, no evidence of adherence of cells to the endothelial lining was noted. In Figure 3b, the dispersion of the individual collagen fibers is more clearly demonstrated. In addition, several areas of osmophilic smudging are visible.

The presence of a large number of endothelial pinocytotic vesicles indicating increased endothelial lining activity is shown in Figure 3c. The width of the thin portion of the alveolar capillary membrane generally remained unchanged after hemorrhagic shock. In an occasional section, it was widened from the normal 800 Å to approximately 1000 Å. The effect of hemorrhagic shock on the morphology of the Type II cell is shown in Figure 3d. These cells show no obvious changes from the control state. The surface microvilli appear normal, the cell wall is intact, the mitochondrial matrix is unchanged, and the osmophilic lamellated bodies, which are presumably surfactant precursors, have retained their normal appearance.

An explanation for the development of interstitial pulmonary edema in the absence of endothelial injury in shock is not readily apparent. If the endothelial layers were injured during hemorrhagic shock, the most plausible explanation for edema would be excess amounts of fluid leaving the capillary by way of an injured lining layer. Since this layer does not appear damaged, another explanation for the interstitial edema must be sought.

A possible explanation for the development of interstitial edema is related to changes in the concentration of interstitial sodium. It has been shown by Moyer (14) and Fulton (15) and their co-workers that collagen develops a heightened affinity for sodium and water following various kinds of stress. Reduction in pH is especially potent in producing this effect.

Further evidence that substantial amounts of sodium may be taken up in the lung under conditions of stress was recently presented by Gump and his associates (16). They carried out a study in humans in which a bolus contain-

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FIGURE 1a. Araldite-embedded section of a normal alveolar capillary region (0.5–1 μ thick) in a baboon lung stained with toluidine blue and Safranin O. This shows a blood vessel containing a number of red blood corpuscles and a white cell. The flattened nucleus of an endothelial cell can be seen in the left corner. The integrity of the endothelial lining is intact. The collagen is stained pinkish-red, and the dark-red strands represent reticulum fibers. The metachromatically stained cells in the top are pneumocytes; however, the types cannot be discerned. X400, in oil.

FIGURE 1b. Another view of the alveolar capillary region in a normal lung. This view focuses on the shape, size, compactness, and spread of the red collagen fibers. With the use of a magnifying glass in some areas the silhouette of bundles of collagen fibers are discernible. Note the blood vessels with the endothelial lining, and the pneumocytes scattered throughout. X400, in oil.

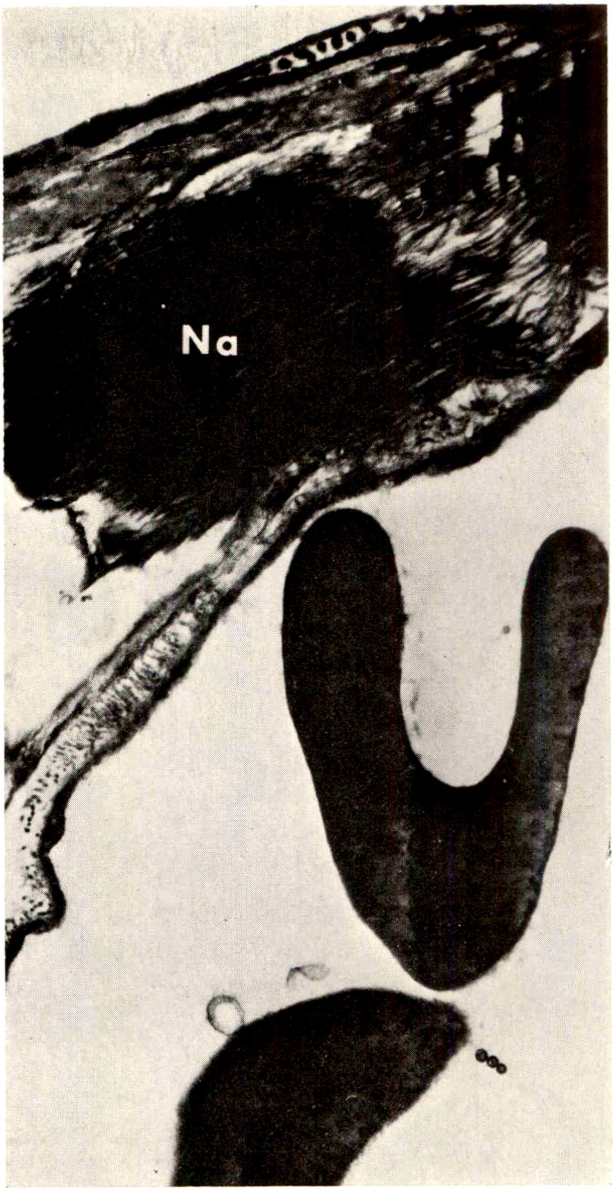

FIGURE 12. A section through the alveolar capillary membrane following hemorrhagic shock and albumin resuscitation. Note the persistence of interstitial sodium pyroantimonate precipitation and edema. ×20,286.

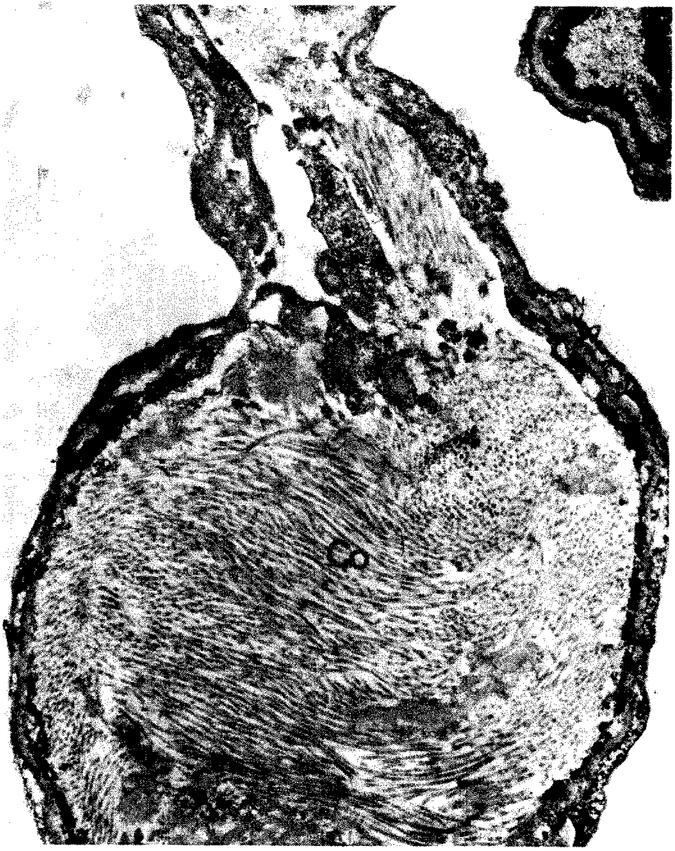

FIGURE 11. A section through an alveolar capillary membrane following hemorrhagic shock and saline resuscitation. Note the normal distribution of collagen fibers, normal distribution of sodium pyroantimonate precipitation, and minimal degree of smudging. ×15,900.

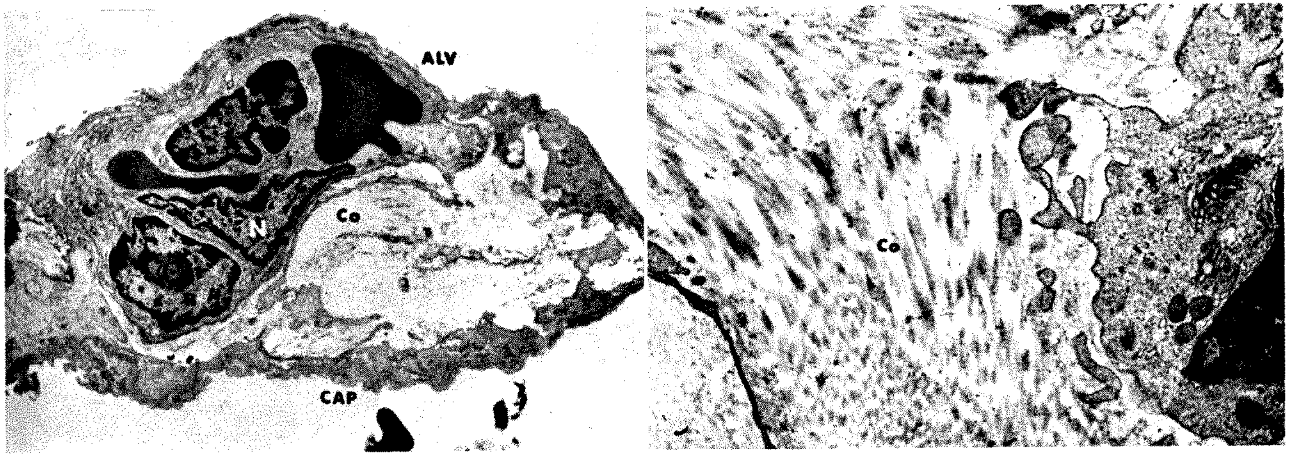

FIGURE 10. In the left panel is a low-power section through the alveolar capillary membrane following hemorrhagic shock and resuscitation with 5 percent albumin. Note the dispersion of the collagen fibers. The right panel is a high-power view of the collagen fibers demonstrating edema of individual fibers and dispersion between fibers.

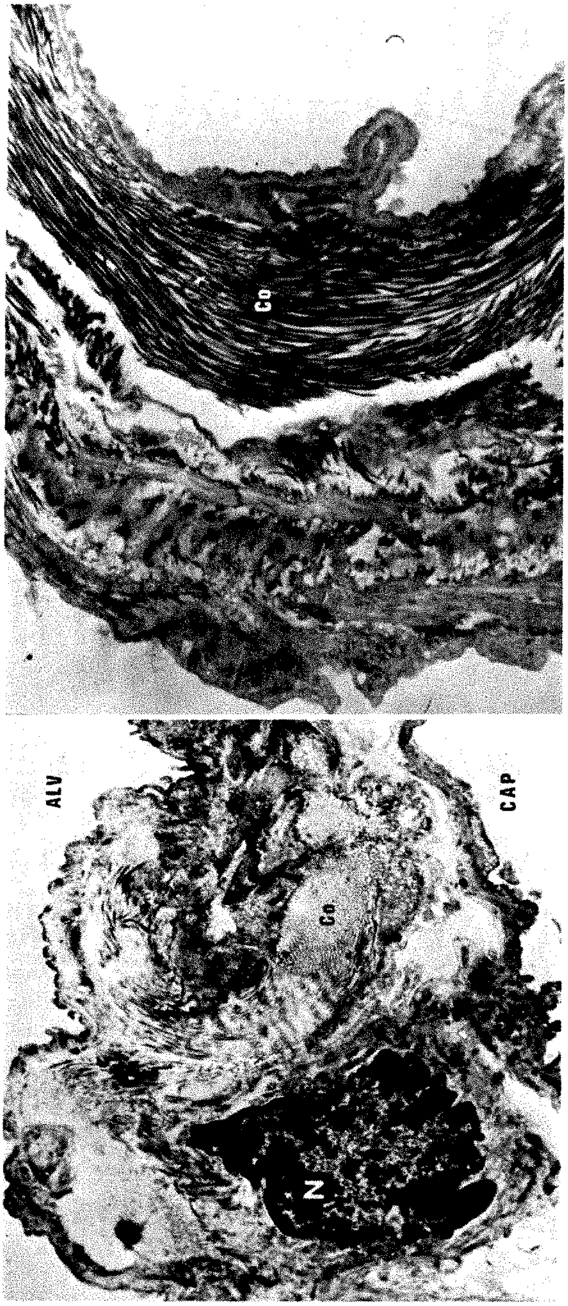

FIGURE 9. In the left panel is a low-power view of an alveolar capillary membrane following hemorrhagic shock and resuscitation with saline. Note the normal appearance of the collagen fibers. The right panel is a high-power view of the collagen fibers. They are deeply stained, and tightly packed.

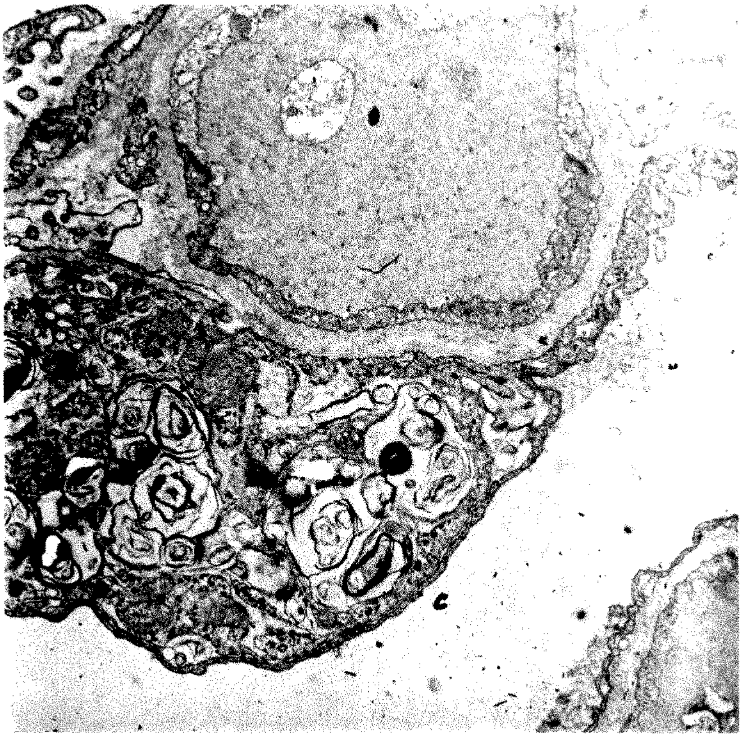

FIGURE 8d. An electron photomicrograph of an alveolar Type II pneumocyte from a dog lung following hemorrhagic shock. This cell demonstrates disorganization of the laminated bodies contained in the cytoplasm, which supposedly are the precursor sites for surfactant production. The mitochondria are swollen and distorted. Glycogen particles are present in the cytoplasm in increased number. Adjacent endothelial lining of the capillary is morphologically abnormal and interstitial edema is present in the connective tissue space. Cell swelling in the endothelial and epithelial cells is found early in hemorrhagic shock. Magnification: ×28,500. Courtesy of Dr. James Wilson.

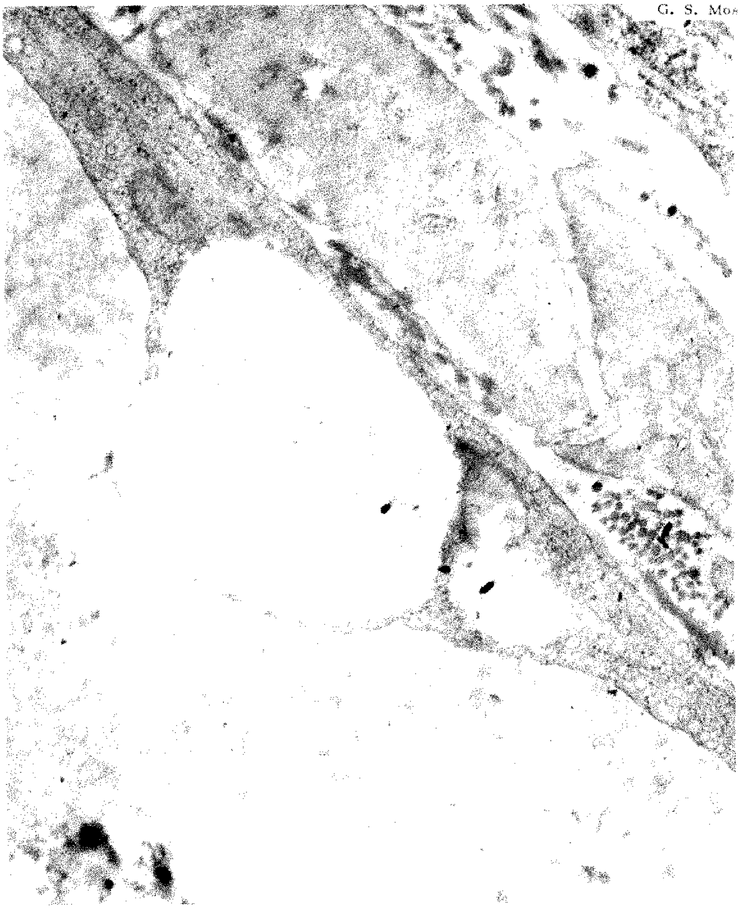

FIGURE 8c. An endothelial lining cell which is disrupted and edematous. A large bleb formation in this cell has protruded into the lumen and the adjacent mitochondrion is damaged. Interstitial edema is also present in this section, even though it is minimal after only one hour of hemorrhagic shock. This, again, is dog lung. Magnification: ×50,500. Courtesy of Dr. James Wilson.

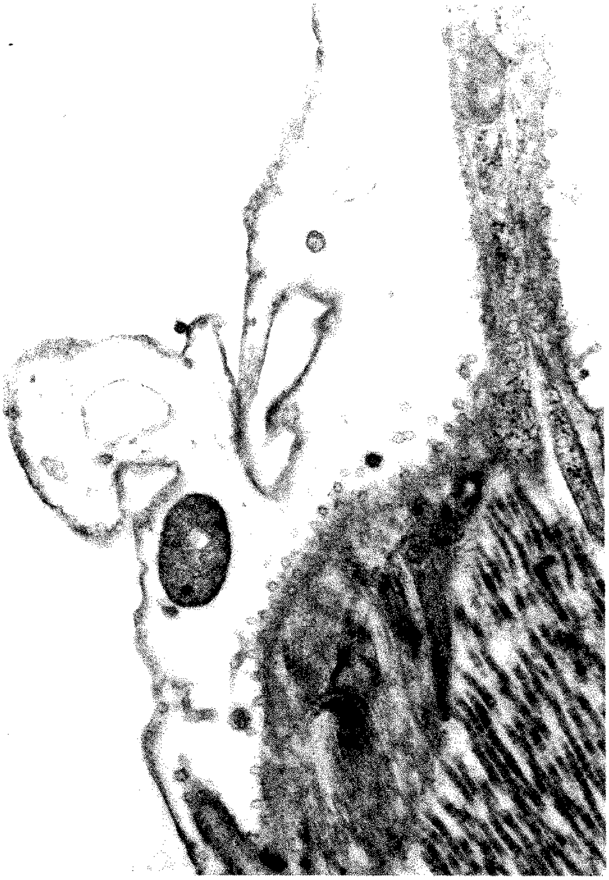

FIGURE 8b. An alveolar Type I epithelial lining cells from the dog lung in hemorrhagic shock. The cell cytoplasm is swollen, the distance from the basement membrane to the plasmalemmna is increased, and the pinocytotic vesicles are lined up along the basement membrane. A mitochondrion is present in this cell and its christi system is morphologically normal. The collagen fibrils at the lower left show a normal periodicity. This type of cytoplasmic swelling is characteristic of early cellular changes in hemorrhagic shock. Magnification: ×52,000. Courtesy of Dr. James Wilson.

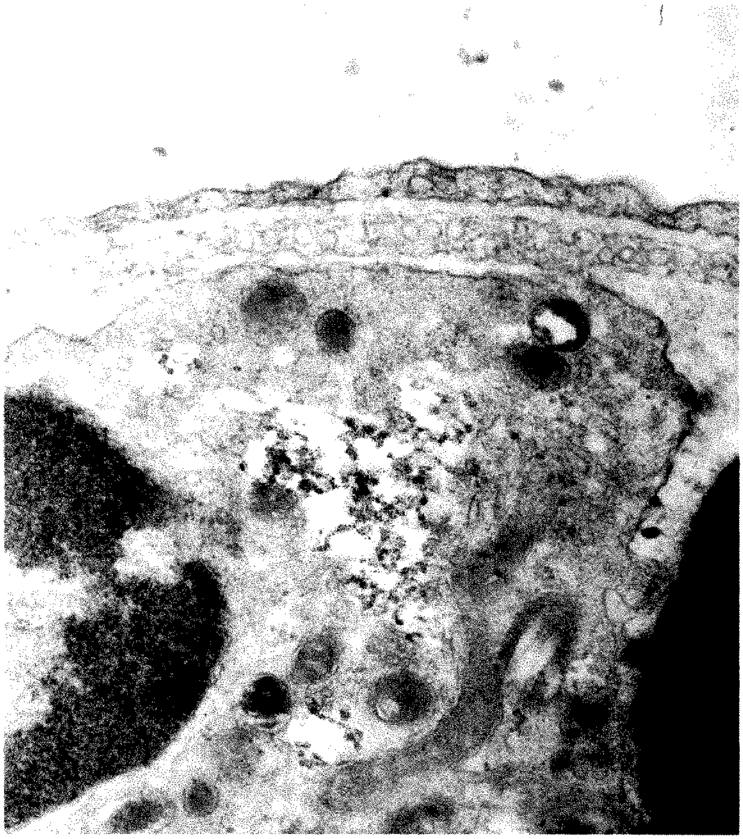

FIGURE 8a. A high-power electron photomicrograph of a polymorphonuclear leukocyte tightly apposed against the endothelium of a small pulmonary vessel in the lung of a dog in hemorrhagic shock. The lysosomes have lost their density, glycogen deposits are present within the cell cytoplasm, and the characteristic space between the limiting membrane of the white cell and the plasmalemma of the endothelium is compromised. The alveolar epithelial cell is relatively normal as is the interstitium. The black area in the lower right is an adjacent red blood cell. Magnification: × 43,700. Courtesy of Dr. James Wilson.

tion, interstitial edema, and damage to the granular pneumocytes. These findings suggested that hemorrhagic shock and resuscitation with whole blood in the cat are associated with diffuse injury of all the components of the alveolar capillary membrane. Studies by Barkett et al (25) in dogs, and in our laboratory in baboons, revealed a somewhat different result. In the dog, retransfusion of the shed blood was followed by the electron microscopic appearance of interstitial edema. No evidence of injury to other elements of the alveolar capillary membrane was noted.

In the baboon, retransfusion of the shed red cells plus infusion of a volume of isotonic saline necessary to restore prehemorrhage arterial pressure values, produced no detectable pulmonary changes at all by electron microscopy (26). Figure 9 demonstrates the alveolar capillary membrane of a baboon after 2 hr of hemorrhagic shock resuscitated with the shed red cells plus 150 cc/kg of isotonic saline. Note the normal distribution of the collagen fibers. In other studies, direct measurement of lung water following shed red cells plus saline resuscitation (150 cc/kg) failed to reveal elevations in pulmonary lung water content above control levels. These findings indicated that after a period of hemorrhagic shock, the primate lungs can tolerate the volume of noncolloid fluids required for resuscitation. Naturally, if volumes greater than necessary are given, pulmonary edema can be expected.

If colloid solutions are used rather than simple salt solutions for resuscitation along with shed red cells, a different ultrastructural pattern can be seen in the baboon lung. Figure 10 shows the alveolar capillary membrane of a baboon after 2 hr of hemorrhagic shock resuscitated with shed red cells plus 50 cc/kg of 5 percent albumin in saline. Definite changes from the control state can be seen. The principle finding is increased interstitial edema surrounding collagen fibers in the thick portion of the alveolar capillary membrane. No other major changes were seen. These changes suggest that infused albumin molecules may leave the pulmonary vascular space during hypovolemia and become entrapped in the pulmonary interstitium, thus favoring the movement of plasma water across the pulmonary capillary.

A comparison of pulmonary sodium interstitial distribution after either saline or 5 percent albumin in saline resuscitation plus shed red cells again reveals a major difference in these two modes of resuscitation. The alveolar capillary membrane from saline-resuscitated animals reveals a normal pattern of interstitial sodium distribution (Fig. 11). In the albumin-resuscitated animals, the alveolar capillary membrane resembles the appearance seen in the shock state, i.e., a substantial degree of sodium-rich interstitial edema surrounding collagen fibers (Fig. 12).

Thus, the comparison of saline versus albumin resuscitation suggests that saline resuscitation is associated with lungs that appear normal ultrastructurally while albumin resuscitation is associated with the persistence of sodium-rich interstitial edema noted in the shock state. Whether or not these differences are important physiologically or clinically remains to be seen.

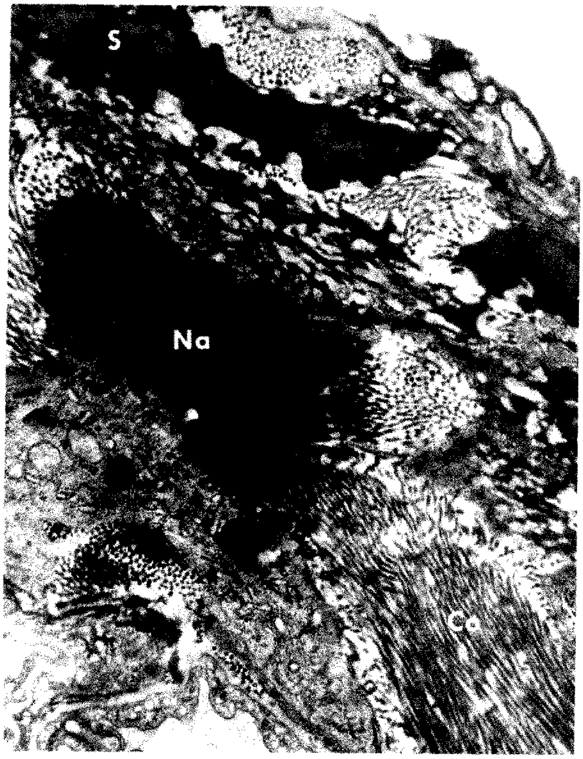

FIGURE 7. Higher power view of one area showing the deposition of sodium pyroantimonate as well as smudging. ×26,180.

alveolar pulmonary edema, a condition known to develop in man with the post-resuscitation pulmonary insufficiency syndrome.

MORPHOLOGIC CHANGES ASSOCIATED WITH RESUSCITATION

An area of intense interest to many investigators has been the relationship between fluid replacement and changes in pulmonary structure and function since most instances of post-trauma or shock pulmonary insufficiency develops following massive fluid therapy. Studies have explored the effects of whole blood transfusion, albumin infusion, and salt solution infusion.

The effect of acute transfusion following hemorrhagic shock in the dog has been studied by Ratliff and his associates (24). They noted, as shown in Figures 8a through 8d, profound changes in the ultrastructure of the cat pulmonary tissue, including injury to both endothelial and epithelial layers, adherence of platelets to the damaged endothelium plus platelet agglutina-

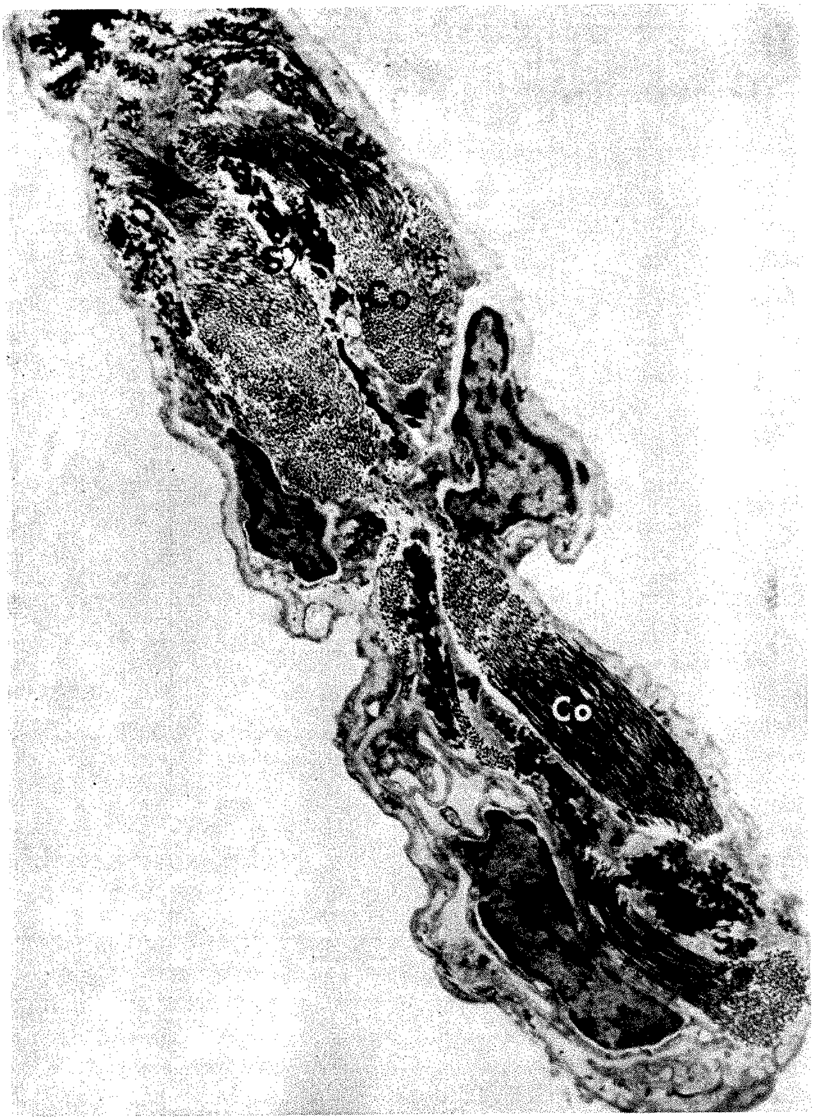

FIGURE 6. A low-power view of a lung in hemorrhagic shock. Note the distribution of sodium pyroantimonate deposits, as well as the smudging in different areas. ×15,370.

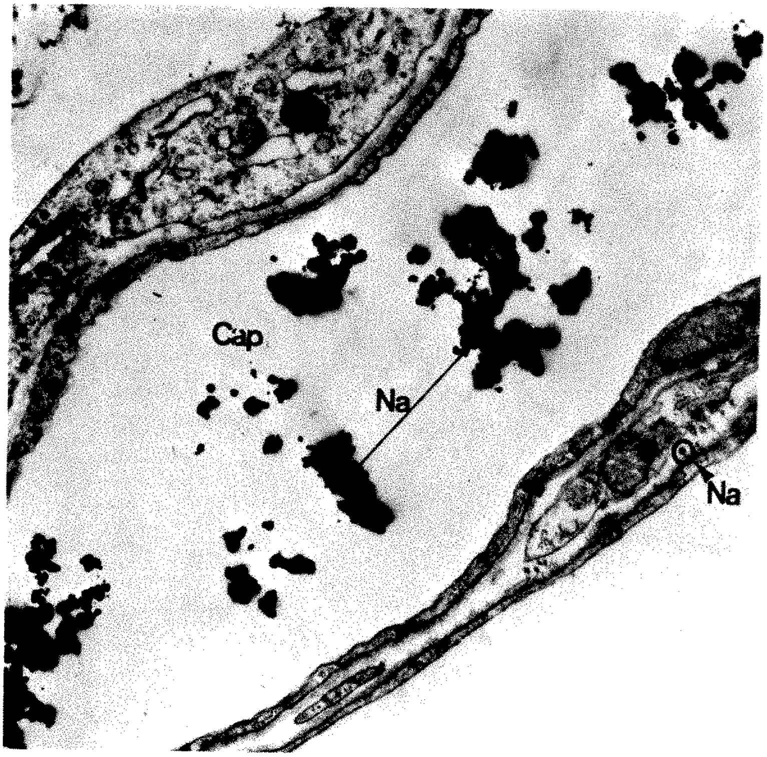

FIGURE 5. Cross section through alveolar capillary membrane in a normal lung. Electron-opaque sodium pyroantimonate deposits are seen within the lumen. Also note the random dispersion of the material throughout the interstitial zone. ×25,200.

excess interstitial water and thereby maintain the alveoli in a dry state. The fact that there is a delay in lymph flow from the lung during overinfusion-induced pulmonary edema supports the idea of initial water binding by collagen.

The findings of dispersion of individual collagen fibers after hemorrhage plus a major increase in pyroantimonate precipitates in these same areas may suggest at least a partial explanation for the development of post-resuscitation pulmonary insufficiency often seen in patients suffering from severe trauma or hemorrhage. It is possible that the high concentrations of sodium developing in the interstitium during the shock produce local osmotic gradients, which results in the transudation of plasma water into the interstitium, and subsequent saturation of collagen-water binding sites. During resuscitation, such sites could no longer function in a normal fashion to aid in the maintenance of dry alveoli. The result could be interstitial and

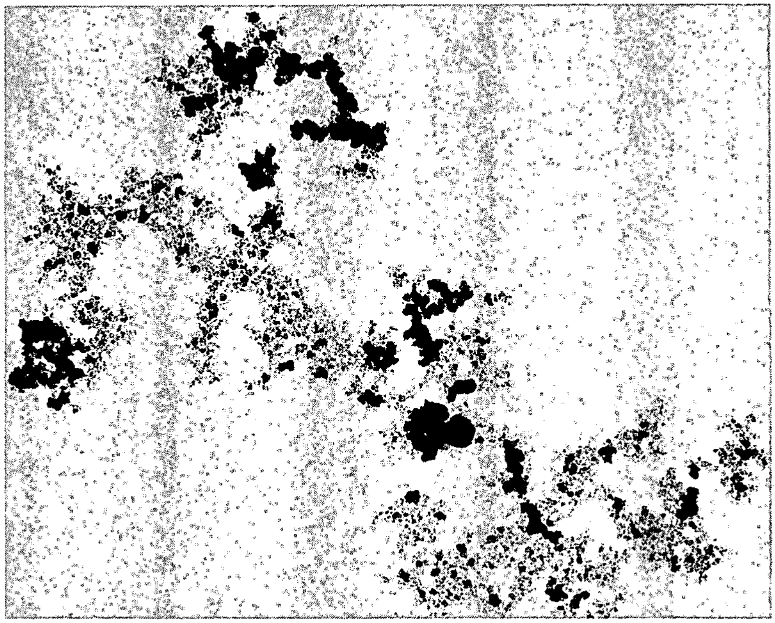

FIGURE 4. Sodium pyroantimonate precipitate. This was obtained by adding 1 cc of perfusing fluid to 9 cc of 0.9 percent NaCl solution. ×35,060.

sodium is the principal extracellular cation. 3. If tissues are harvested in a solution high in sodium content prior to fixation with pyroantimonate, there is a qualitative increase in interstitial precipitates (19). 4. If specimens of glutaraldehyde-fixed lung tissue are kept in a sodium-free phosphate buffer solution for five days prior to pyroantimonate treatment, only negligible amounts of precipitates can be seen (19). 5. Electron microscope autoradiography of kidney tissue has shown that the precipitates contain sodium (21). 6. In tissues fixed with pyroantimonate, radioactive sodium ions show a slower efflux than that seen in controls (22). For these reasons, Torack & La Valle (20) have recently concluded that extracellular localizations of sodium ion by this technique should be considered to be a valid representation of this cation.

The studies of the lung morphology during hemorrhagic shock emphasize the potential importance of the pulmonary collagen fibers. Originally, they were thought to function only as a structural framework for the lung. Cottrell et al (23), in an ultrastructural study of hemodynamically induced pulmonary edema, pointed out that pulmonary edema begins first in the thick portion of the alveolar capillary membrane in the areas occupied by collagen fibers. He proposed that these fibers might function as sponges to pick up

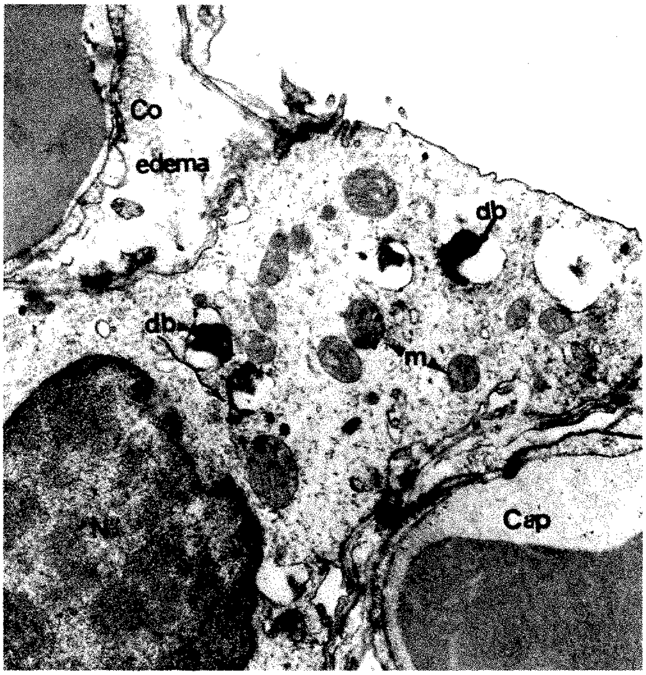

FIGURE 3d. A cytoplasm of a Type II cell in a baboon lung following hemorrhagic hypotension. Note that the cytoplasm does not show any apparent difference from a control Type II pneumocyte (Fig. 2a) (db = lamellated dense bodies, m = mitochondria, co = collagen and cap = capillary). ×20,894.

collagen fibers, the heavy concentrations of electron-dense particles in the areas occupied by the collagen fibers, and the smudging of the sodium pyroantimonate in those same areas.

Critique of the sodium-marking technique.—Since the pyroantimony technique was originally described in 1962, the specificity and sensitivity of this method of sodium marking has been tested by a number of investigators. The most serious problem was identified by Bulger (19) who showed in in vitro studies that precipitates could be formed with other cations including potassium, magnesium, and calcium. Since all three cations are found in relatively high concentrations within cell cytoplasm, interpretations of intracellular precipitates must be made with caution.

On the other hand, extracellular precipitates appear to be primarily sodium pyroantimonate. The evidence for this is as follows: 1. pyroantimonate is approximately 10 times more sensitive for sodium than the other cations (20). 2. While other cations are present in low concentrations, through both the vascular and the endobronchial systems in order to insure even fixation and to minimize distortion during processing.

The results of mixing an equal volume of aqueous potassium pyroantimonate with isotonic saline is shown in Figure 4. The insoluble precipitate, sodium pyroantimonate, is visualized as electron-dense particles. A similar pattern will be seen in pulmonary tissue fixed with this solution.

A cross section through the alveolar capillary membrane of a normal lung fixed with potassium pyroantimonate is shown in Figure 5. Electron-dense particles representing sodium pyroantimonate can be seen in the capillary lumen. In addition, similar particles are randomly scattered throughout the interstitial zone.

The effects of 2 hr of hemorrhagic shock on sodium distribution in the primate lungs are shown in Figure 6. The features noted in Figure 3 are again seen, i.e., edema surrounding the individual collagen fibers. In addition, there is a major increase in the number and concentration of electron-dense particles in the interstitium surrounding the collagen fibers. Smudging of the sodium pyroantimonate is also prominent in these areas. Figure 7 is a higher power view of the same area illustrating the dispersion of the individual

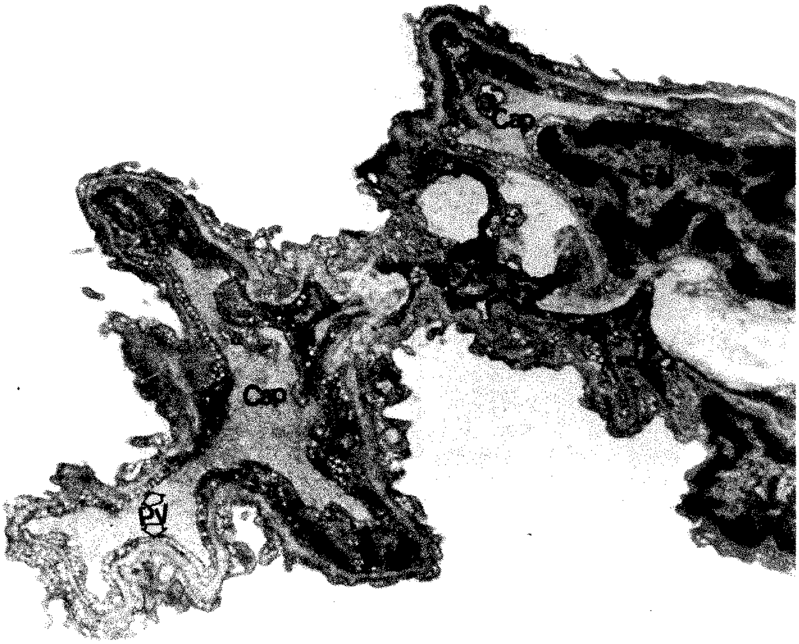

FIGURE 3c. Higher power view of a capillary. Note the marked increase of the pinocytotic vesicles (PV). ×17,290.

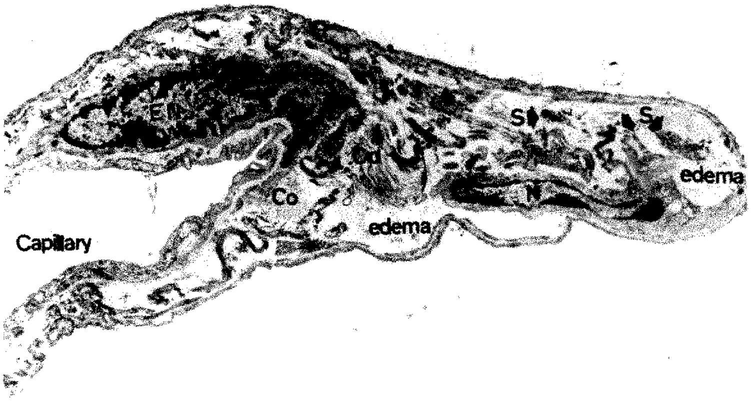

FIGURE 3a. Low-power view of the alveolar capillary membrane of a baboon lung in shock. Note the general disarray in the interstitial space. Considerable edema is apparent and the collagen fibers (CO) are noted. Furthermore, there is an increase in the number of pinocytotic vesicles in the capillary endothelial lining. Both smudging and vesicles will be more prominent in higher power (see below). A pneumocyte nucleus (N) is seen, but the type cannot be identified in this magnification. ×7,650.

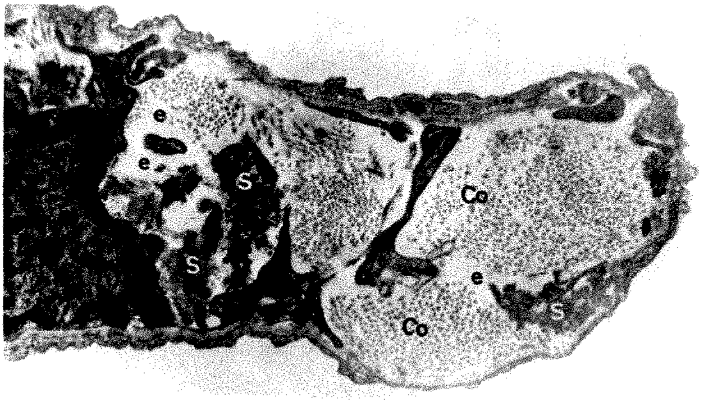

FIGURE 3b. Higher power showing the edema (e), dispersion of the collagen fibers (CO), and osmophilic smudging (S) in the alveolar capillary region of a lung in shock. In the left part of the micrograph a portion of a nucleus of an endothelial cell is seen. These smudged areas correspond to the "pool" seen in light microscopy and represent areas of edema. ×22,900.

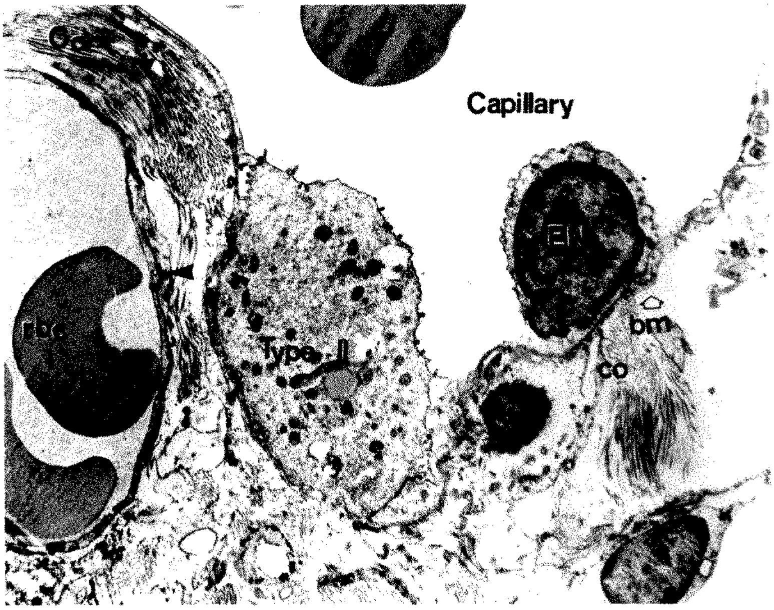

FIGURE 2a. A low-power view of the alveolar capillary region of a normal baboon lung. The capillary lumen with red blood cells are obvious. The endothelial nucleus (EN) is marked in one capillary. A Type II pneumocyte can be easily identified. The collagen fibers (CO) are marked and the distribution in the interstitium is apparent. Basement membrane (bm) is only marked in one area and the arrow points to a pinocytotic vesicle. ×7500.

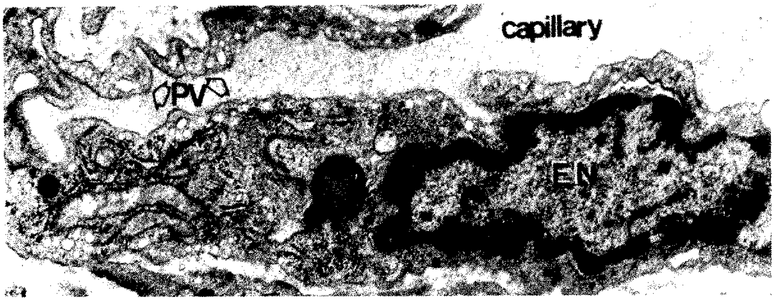

FIGURE 2b. Higher power of a capillary. Note the presence of pinocytotic vesicles (PV). The endothelial nucleus is labeled EN. ×52,360.

ing radioactive sodium, radioactive iodine, and tritiated water was injected simultaneously into a central vein and collected at frequent intervals from a peripheral artery. In control studies, approximately 95 percent of the injected sodium was recovered in a single pass through the lungs. Under conditions of stress, an average of only 85 percent of the sodium could be recovered in a single pass, indicating that take-up of sodium in lung tissue had occurred. These observations following shock of pulmonary interstitial edema plus sodium take-up in the lung coupled with the fact that a substantial amount of collagen is present in the interstitial area of the alveolar capillary membrane leads one to the following hypothesis. One of the major effects of hemorrhagic hypotension is the development of metabolic acidosis. This change in pH results in a heightened affinity of pulmonary interstitial collagen for ionic sodium. Increased numbers of sodium ions traverse an intact pulmonary endothelial layer possibly by way of pinocytotic vesicles and become adsorbed to the collagen fibers. This produces a local increase in tissue osmolarity which rapidly results in the movement of water into the areas occupied by the collagen fibers. The net result is interstitial edema.

In order to test the hypothesis that increased amounts of sodium accumulate in areas of the pulmonary interstitium occupied by collagen fibers following hemorrhagic shock, it is necessary to be able to identify sodium distribution, preferably by ultrastructural histochemical techniques. Such a technique for sodium localization was first proposed by Komnick et al (17) in 1962 and was subsequently tested in a number of different tissues. The method involves the fixation of the tissue in question with an aqueous solution of unbuffered potassium pyroantimonate. Such a solution possesses two important characteristics; (*a*) the pyroantimonate ion has a great affinity for sodium, and (*b*) the resulting complex, sodium pyroantimonate, is insoluble and appears as electron-opaque particles by electron microscopy. Recently, Das Gupta et al (18) modified this technique for use in primate lung tissues. The method utilizes in vivo dual simultaneous fixation

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FIGURE 1c. 0.5 to 1 μ Araldite-embedded section from the alveolar capillary region of a baboon lung after hemorrhagic hypotension. The red collagen fibers are seen to be wavy and disrupted, and the compactness of the collagen bundles is not as apparent as in the control sections. In the lower central part of the section, the disarray is obvious. However, the individual pneumocyte seems to have maintained the intracellular integrity. There is no loss of integrity in the endothelial lining. ×400, in oil.

FIGURE 1d. The destruction of the integrity of the collagen bundles and edema in the interstitial space is obvious. In this area, the collagen fibers are not stained intensely and appear to be ending in a "pool." These represent edema. Again, intracellular integrity and integrity of the endothelial lining are not disturbed. ×400, in oil.

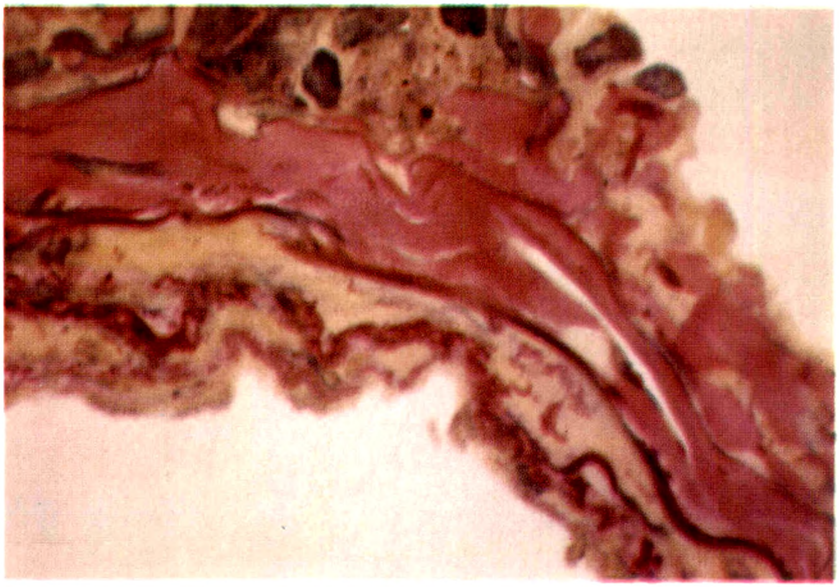

FIGURE 1c

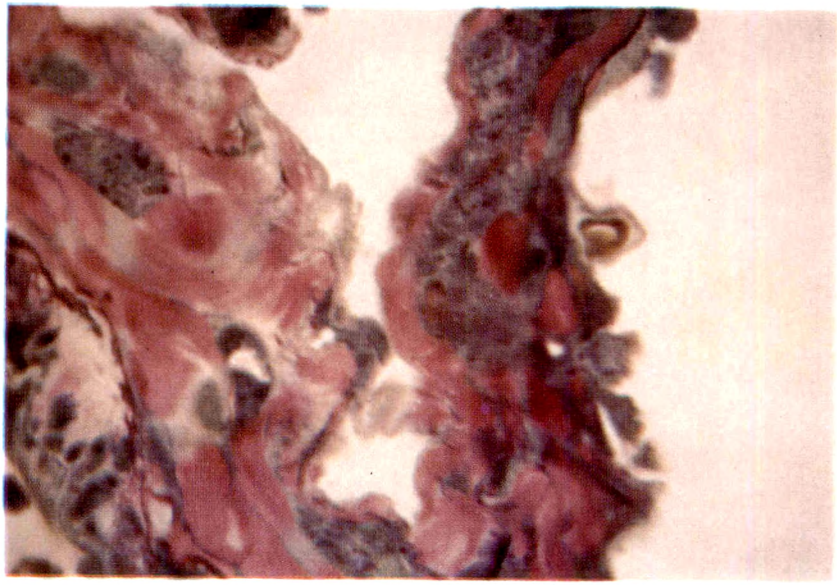

FIGURE 1d

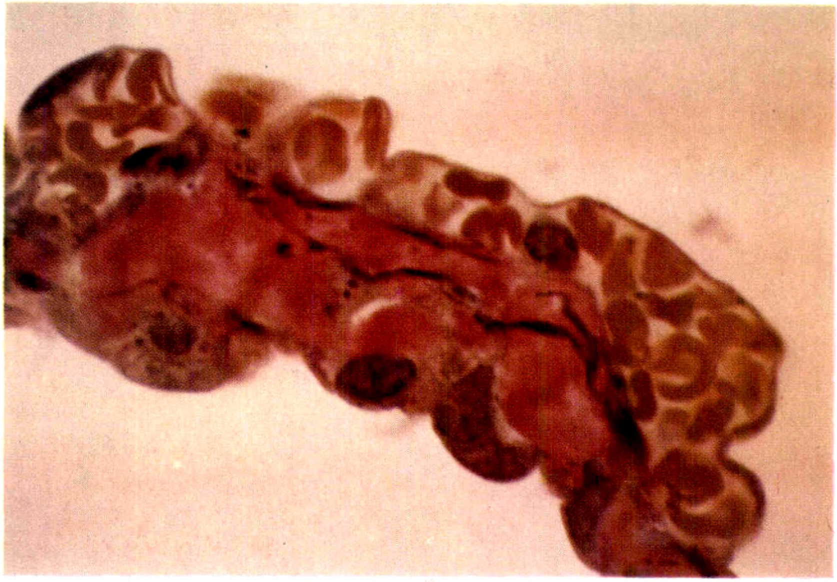

FIGURE 1a

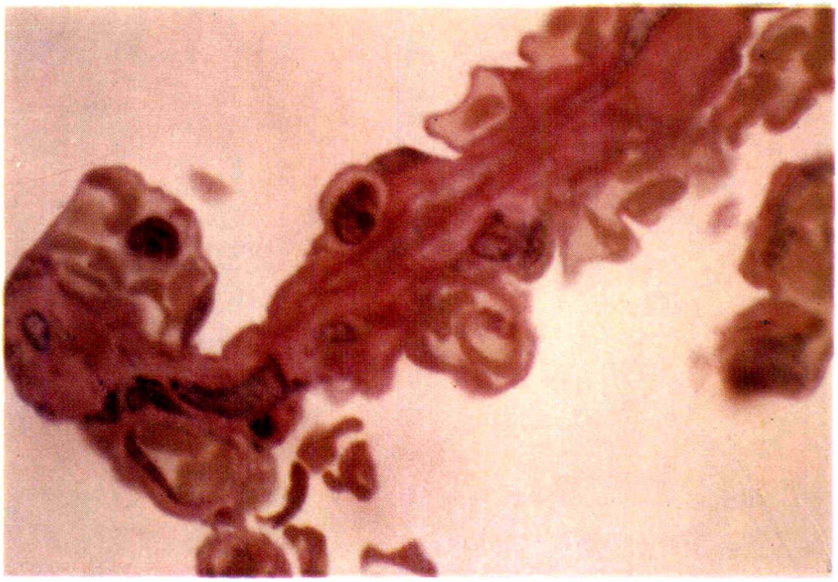

FIGURE 1b

SUMMARY

Pure hemorrhagic shock is well tolerated physiologically by the respiratory system. However, ultrastructural studies have revealed major changes in the alveolar capillary membrane. The principal change is the development of sodium-rich edema fluid in the thick portion of the alveolar capillary membrane. Studies following resuscitation indicate that from a morphologic point of view, red cells and salt solution are associated with return of the alveolar capillary architecture to normal while either whole blood transfusions, or red cells plus albumin solution are associated with residual abnormalities in the alveolar capillary membrane.

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SURGICAL PROCEDURES FOR CORONARY ARTERY DISEASE

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Coronary atherosclerosis is a biochemical disease of uncertain etiology which produces myocardial ischemia from mechanical obstruction of the coronary arteries. Operative procedures are designed to improve the flow of blood to ischemic areas of myocardium, but, of course, do nothing to alter the underlying biochemical abnormality. Hopefully, dietary and other therapeutic procedures will be found to prevent progression of the process and bring about possible improvement in coronary atherosclerosis; however, significant data are not yet available. Hence, at present, operative procedures which directly increase myocardial blood flow offer one of the best methods of therapy for patients seriously disabled with coronary disease.

Since the introduction of bypass grafting for coronary artery obstruction in 1967–1968, scarcely four years ago, probably more experience and interest has accumulated with direct surgical procedures for coronary artery disease than in the entire span of time since Beck began his initial attempts to revascularize the heart between 1935 and 1940. Most discussion in this review will be concerned with bypass grafting, this procedure having become the mainstay of operative therapy for most patients. Arterial implants and excision of ventricular aneurysms or scars will be mentioned briefly.

HISTORICAL CONSIDERATIONS

In 1937, both Beck and O'Shaughnessy (1, 2) reported ingenious attempts to improve myocardial blood supply by suturing different structures to the myocardium, often in combination with the introduction of various irritants, such as talc, to induce formation of adhesions. At a later date, other operative procedures included arterialization of the coronary sinus and partial ligation of the coronary vein. Unfortunately, none of the operations withstood the test of time and are now of historical interest only. The tendency for collagen in scar tissue to contract progressively and become more ischemic is probably the basic biologic reason that operative procedures which depended upon the vascularity of adhesions were unsuccessful.

The ingenious concept of arterial implants was developed by Vineberg in the laboratory in 1946 and subsequently applied clinically in 1950 (3). Only limited clinical experience was available until Sones demonstrated with coronary angiography in 1956–1957 that the implanted internal mammary artery remained patent and dye injected into the internal mammary artery would opacify regional coronary vessels. Until the development of bypass grafting, implants were the most commonly performed operative procedure for coronary disease and an extensive clinical experience accumulated in several centers between 1963 and 1968. The degree of clinical improvement, however, has been far inferior to that obtained with successful bypass grafting; so, arterial implants have greatly waned in popularity in the past three years.

Direct removal of atherosclerotic plaques by coronary endarterectomy was closely studied by Cannon and Longmire et al (4) and by Sabiston & Blalock (5). Initial results were prohibitively discouraging because of the high operative mortality and the subsequent high percentage of late occlusions. Subsequently, endarterectomy was combined with patch-grafting to widen the arterial lumen, and was extensively applied at the Cleveland Clinic. The technique was tedious, especially for areas of occlusion greater than 2 cm in length, and late occlusion developed in a significant percentage of these patients. Dissatisfaction with the patch-graft reconstructive technique led Favaloro to utilize short segments of saphenous vein for localized obstruction of the right coronary artery (6). After this initial step, longer and longer segments of saphenous vein were employed, initially for longer segments of the right coronary artery and then for the anterior descending and circumflex coronary arteries. Additional contributions to the technique of bypass grafting in 1967–1968 were made by Johnson and associates (7) at Marquette University in Milwaukee and by Adam, Mitchel & Lambert in Dallas (8).

At New York University Medical Center, Green et al (9) first utilized the internal mammary artery as a direct end-to-side anastomosis to the left anterior descending coronary in February 1968. The only previous report of use of the internal mammary artery for direct anastomosis was by Kolessov in 1967 (10), although several experimental studies had evaluated this possibility (11). The relative choice between internal mammary artery grafts and saphenous vein grafts is considered further in the section on operative technique.

BYPASS GRAFTS

The belated discovery of the widespread feasibility of bypass grafting is surprising. As recently as 1967 most operative procedures upon the left coronary artery had a prohibitive mortality, exceeding 50 percent (12). Now, bypass procedures are performed upon the left coronary artery with an operative risk of less than 5 percent. The various factors responsible for this successful application of bypass grafts include the immediate increase in flow of blood to ischemic myocardium, technical advances in microsurgery of small arterial anastomoses, excellent angiography, and the increasing safety of all operative procedures performed with cardiopulmonary bypass.

Bypass grafts have been used principally for patients with angina pectoris. With the encouraging results obtained, bypass grafts have been tried for some patients with congestive heart failure, acute myocardial infarction, and preinfarction angina. In the following sections, most discussion concerns elective bypass grafting for angina pectoris, but experiences thus far available with bypass grafting for the other three manifestations of coronary atherosclerosis will be briefly mentioned.

BYPASS GRAFTING FOR ANGINA PECTORIS

Rationale of operation.—Bypass grafting is possible because occlusive atherosclerotic plaques develop principally in the proximal one-third or one-half of the major coronary arteries, but distal vessels remain patent in the majority of patients. In an extensive study of cadaver hearts, Schlesinger & Zoll (13) found that 69 percent of coronary occlusions were located in the proximal 4 cm of the main coronary arteries. In a series of nearly 50 atherosclerotic hearts examined by Green with severe disease in the proximal left anterior descending coronary, the artery was patent distally in over 90 percent (14). Similarly, Berger (15) studied nearly 300 postmortem hearts with coronary atherosclerosis and found a patent segment beyond the area of obstruction in 80 to 90 percent of patients. This high frequency of patent distal vessels has been long noted by pathologists, but the clinical significance was not recognized. This is due partly to the small size of the arteries in the contracted, postmortem heart, and also to the fact that the contracted vessel distal to an area of obstruction can be readily dilated with small graduated probes, often to more than 50 percent of its initial diameter.

Localization of coronary atherosclerosis to proximal coronary arteries has also been confirmed by many angiographic studies. In a series of consecutive coronary angiograms performed on patients with angina at New York University, Glassman reported that 52 of 54 patients were suitable candidates for bypass grafting (16). Criteria of operability, however, have varied in different centers. Operability is naturally less if the patients are chosen only if a significant coronary vessel, 2 mm or greater in diameter, can be seen distal to the area of obstruction. On the other hand, operability is much greater if patients are accepted on the basis of simple angiographic demonstration of proximal occlusion. At New York University, the latter criterion has been used, for the vast majority of patients at operation will be found to have a patent distal segment, although it may be only in the range of 1 to 2 mm in diameter. If patients are excluded from operation simply because a vessel cannot be visualized, a number of patients would be excluded who can readily benefit from operation. With these liberal criteria, it seems clear from data from many sources that over 90 percent of patients with angina pectoris are technically feasible candidates for bypass grafting (6, 14–16).

Selection of patients for operation.—Operation is usually considered for disabling angina pectoris that is unresponsive to drug therapy. Policy varies among different medical centers, depending upon experience and enthusiasm. Hence, the available data vary somewhat with regional patient selection. Factors influencing the decision for operation include age of the patient, duration of progression of angina, and ability of the patient to function at normal activities with drug therapy. Age, per se, has not been a significant factor. The oldest patient operated upon at New York University, 73 years of age, obtained prompt and lasting relief from severe, crippling angina pectoris following a single bypass graft to the left anterior descending coronary artery. Coronary angiography is initially performed to determine the localization and extent of the obstructive lesions. A stenosis greater than 50 to 60 percent of the normal diameter of the artery is considered physiologically significant. As mentioned in the preceding paragraphs, with present criteria, over 90 percent of patients with angina pectoris are potential candidates for bypass grafting.

A major contraindication to operation is congestive failure with extensive destruction of left ventricular muscle. Ventriculography in such patients demonstrates impairment of myocardial contractility, resulting from destruction of large areas of ventricular muscle and subsequent replacement by fibrous tissue. The evaluation of ventriculograms is not a precise technique, however, and akinetic areas may function reasonably well after bypass surgery, apparently representing nonfunctional but viable muscle. The presence of congestive heart failure in association with extensive impairment of contractility is an ominous sign, for little improvement may follow bypass grafting. Left ventricular end diastolic pressure is another indication of severity of congestive failure. Mitral regurgitation demonstrated on left ventricular angiography is an equally ominous sign. It usually results from papillary muscle dysfunction because of extensive muscular disease of the left ventricle.

At present, bypass grafting is usually not recommended on a "prophylactic" basis in patients who are functioning well but have significant coronary arterial disease demonstrated on angiography. It may be found with more long-term data that bypass grafting is indicated to protect against future myocardial infarctions, but more data are needed before this question can be answered.

Operative technique.—Almost all bypass grafts are performed with cardiopulmonary bypass to still the heart, either by induced ventricular fibrillation or cardiac arrest, while performing anastomoses to 1 to 3 mm vessels. Perfusion even for 3- to 4-hr duration in patients with extensive coronary disease is surprisingly well tolerated, with an operative risk near 5 percent in good risk patients. This is probably true because there is an immediate increase in myocardial blood flow. With congestive heart failure from destruction of left ventricular muscle, operative risk is increased to the range of 15 to 20 percent.

Bypass grafts can be attached to coronary arteries in virtually any area of the heart, including arteries as small as 1.0 mm internal diameter. The anterior descending coronary is usually approached in its midportion. Its diagonal branch may be a significant tributary and require separate exposure. The right coronary artery is usually approached at the origin of the posterior descending; or, alternately in the presence of extensive disease, the posterior descending coronary is approached directly. The circumflex coronary is the most difficult of the three. Often a large tributary of the circumflex is simpler to approach, with subsequent retrograde flow into the main artery.

Multiple grafts are needed in most patients. In the past year triple bypass grafts were performed in nearly 50 percent of patients operated upon at New York University. An important concept evolving from experience has been that involvement of multiple arteries should be treated by multiple grafts, despite the longer operative procedure required. A single bypass graft in patients with multiple areas of atherosclerosis has been found to be inadequate.

In most patients, a saphenous vein has been used as the bypass graft. Green and Tice originally developed the internal mammary technique, first performing the procedure of direct internal mammary-coronary anastomosis in early 1968. Green has continued to use the internal mammary artery in about 80 percent of his patients. The artery is more difficult to use than the saphenous vein, but closely approximates the size of the coronary artery and has the theoretical advantage of an arterio-arterial anastomosis. This theoretical advantage has been confirmed by long-term patency rates greater than 90 percent on angiography performed up to two years after operation, and hence the internal mammary artery may be found to be the graft of choice for small diseased arteries. Its applicability is hampered by the fact that it can be used only for the anterior descending coronary in most patients.

The major limitation of bypass grafting is multiple areas of stenosis in the same artery. Grafting is applicable only for the arterial tributaries beyond the distal stenosis. Gas endarterectomy, developed by Sawyer and colleagues, has been cautiously employed by some groups to remove multiple areas of obstruction. Few data are yet available, but angiographic studies in some patients a year after endarterectomy have shown a disappointing frequency of subsequent stenosis and occlusion, approaching 30 to 50 percent. Hopefully, with experience, better results can be obtained, for currently gas endarterectomy is the only feasible technique for removing multiple areas of obstruction.

Concomitant procedures with bypass grafting include excision of akinetic myocardial scars or left ventricular aneurysms in 10 to 20 percent of patients. These will be mentioned briefly in subsequent sections.

Blood flow in a bypass graft varies with the diameter of the coronary artery and the degree of regional atherosclerosis. Measurements with an electromagnetic flowmeter in the operating room show a corresponding variation in rate of flow, ranging from as low as 20 to 30 ml/min to 90 to 100 ml/min in larger vessels. The average mean flow is between 50 and 75 ml/min. Injection of papaverine (5 to 10 mg) into a bypass graft at operation usually produces a striking increase in flow, often as much as 100 percent. The ability of the distal vascular bed to dilate may have some prognostic significance, as opposed to nonreactive atherosclerotic vessels.

RESULTS

Early results.—In most patients following bypass grafting, angina is promptly and totally abolished. This is a striking observation, unlike that obtained with any previous operative procedures for coronary artery disease. It is almost surely due to the immediate increase in blood flow to ischemic myocardium. Reports from almost all centers reporting bypass grafts have consistently described this observation. In general, angina disappears in 80 to 85 percent of patients and is greatly decreased in severity in the remaining 15 to 20 percent. In almost all of those with residual angina, bypass grafting has not been performed to one of the major obstructed coronary arteries.

Operative mortality has been surprisingly low, less than 5 percent in the good risk patients. This low mortality is particularly surprising because before 1967 attempted reconstructive procedures upon a diseased left anterior descending coronary artery were associated with a prohibitive mortality, exceeding 50 percent. The low mortality is almost surely due to the immediate increase in blood flow through the bypass grafts. Operative risk is partly related to the extent of coronary atherosclerosis, but more directly to the destruction of left ventricular muscle which has occurred, as evidenced by the presence of congestive failure and the impaired contractility on the ventriculogram. In patients with severe impairment of myocardial contractility and congestive heart failure, operative risk may be as high as 20 to 30 percent. In a May, 1971, symposium in Milwaukee, Johnson (17) reported a mortality rate of 3 percent in NYHA Class I patients, 7 percent in Class II, and 19 percent in Class III and IV patients. There were 187 patients in the total group with an overall mortality of 9 percent. Mitchel reported similar results in a group of 400 patients operated upon in Dallas (18). At New York

University the overall mortality in 270 patients operated upon between February 1968 and March 1971 was nearly 10 percent (19). The most extensive experience has been reported from the Cleveland Clinic, where the mortality rate has been near 5 percent in a series of nearly 2000 patients. Patients with severe left ventricular dysfunction and congestive heart failure were usually not operated upon in this series (20).

Long-term results.—With the low operative mortality and the prompt relief of angina as established facts of bypass grafting, one of the most serious questions is long-term patency of vein grafts. Early patency rates evaluated by angiography within three weeks after operation are in the range of 80 to 90 percent (7). The Cleveland Clinic group reported a patency rate of 95 percent is a series of 60 grafts performed between April and June 1970 (20). Early patency rates are determined principally by the size of the vessel selected for anastomosis and the surgical technique. With coronary arteries greater than 2 mm internal diameter, an early patency rate greater than 90 percent can be expected.

The long-term patency of vein grafts is one of the most serious concerns with bypass grafting. In the first year after operation at least 5 to 10 percent of vein grafts occlude. Factors which influence late occlusion include formation of adhesions and perhaps turbulent flow of blood because of discrepancy between the size of the vein graft and the distal coronary artery. At New York University, severe postoperative pericarditis in five patients was associated with the formation of dense pericardial adhesions and obliteration of all vein grafts within four months of operation, resulting in death in three of the five patients. In two other patients, stenosis of a vein graft was found at reoperation to be associated with tension and kinking near the aortic anastomosis, with a resulting focal hyperplasia of the intima and media. This may be the result of adhesions producing localized tension near the anastomosis, a well-known stimulus to proliferation of collagen, familiarly seen in formation of a keloid in operations upon the skin. In virtually all patients in whom there has been a late reappearance of angina, angiography has found a stenosis or occlusion of one or more previously functioning grafts.

Other long-term factors influencing patency are probably related to turbulent flow of blood in the graft. Turbulence may result from tension, angulation, disparity in size of the graft and the coronary artery, or areas of stasis and eddy current formation where venous valves are present. The phenomenon of subintimal hyperplasia reported by Johnson is probably a histologic response to turbulent flow of blood (21). The Cleveland Clinic group has reported an overall patency of 84 percent in a total of 464 grafts studied. Only seven of these, however, had angiograms performed longer than 24 months after operation (20).

Although the present durability of coronary bypass grafts is known to be only one to three years after operation, there are abundant data available regarding venous grafts as arterial substitutes in the lower extremities. Venous grafts have been used intermittently for repair of peripheral arterial injuries for over 50 years, and data are available in a few patients operated upon by Gordon Murray in Canada over 30 years ago. However, most data stem from the extensive use of venous bypass grafts for femoro-popliteal occlusive disease in the past ten years. The five year patency of such grafts, even in the presence of significant distal atherosclerosis, ranges from 70 to 80 percent. Late degeneration to form an aneurysm is virtually unknown (22). Hence, the long-term suitability of a venous graft as an arterial substitute is well established.

Few data are yet available concerning the degree of protection provided by functioning grafts from myocardial infarction and death. Several studies have shown that the expected mortality with angina pectoris ranges between 5 and 10 percent per year, depending upon associated factors such as obesity, hypertension, and diabetes. In the patients operated upon at New York University there have been, to date, no late cardiac deaths in patients with patent double grafts (23, 24). Recurrence of angina or late myocardial infarction in a few patients has invariably been associated with the closure of a functioning graft or development of disease in the left coronary system in patients initially treated only with a bypass graft to the right coronary artery. The Cleveland Clinic group have reported that 90 percent of the first 300 patients operated upon between two and three years earlier are alive with a late infarction rate near 4 percent. A significant number of these patients had grafts only to the right coronary artery (20).

BYPASS GRAFTING FOR CONGESTIVE HEART FAILURE

With the immediate relief of angina with bypass grafting, a natural question has been the value of bypass grafting for congestive heart failure from coronary disease. To date, few data have been published regarding this question. At New York University experiences with 40 such patients were reported in April 1971, representing about 15 percent of a group of 270 patients undergoing bypass grafting. Congestive failure was severe in about one-half of the group. Angina of varying severity was also present in about one-half of the patients. A double bypass graft was performed in 25 and a single graft in 15. In 18 of the 40 patients either a left ventricular aneurysm or a paradoxically contracting myocardial scar was excised. Seven of the 40 also required reconstruction of the mitral valve because of severe insufficiency from papillary muscle dysfunction.

In sharp contrast to bypass grafts for angina, the results were most discouraging. The overall mortality was 37 percent for the entire group of 40 patients. Six died within a few days of operation from an inadequate cardiac output and four others within the first month. Five initially recovered but succumbed within one to ten months after operation, all from progressive congestive failure unimproved by operation. In the surviving patients only eight obtained a good result, as measured by resumption of normal physical activity with few signs of congestive failure. The result was judged as "fair" in 13 others.

Equally disappointing was the fact that cardiac catheterization one to ten months after operation in ten patients found no improvement in the impaired myocardial contractility present before operation. Similarly, left ventricular end diastolic pressure and cardiac output remained unchanged over that present before operation. The bypass grafts were patent in most patients.

An analysis of the different factors which might influence the eventual result showed that intractable congestive failure without angina almost uniformly had a poor result, with a fatality rate approaching 50 percent. If angina pectoris was present as well as congestive failure, prognosis was more favorable. This suggested that the presence of angina indicated ischemic but viable muscle which could be improved with additional supply of blood. There was little difference in outcome between the 18 patients in whom a left ventricular aneurysm or akinetic scar was removed and the 22 patients in whom no segment of the left ventricle was excised. Mitral insufficiency, corrected at operation in seven patients, was uniformly associated with a poor prognosis, over 50 percent of the group dying within a short time. Seemingly, coronary disease extensive enough to produce mitral insufficiency from papillary muscle dysfunction is associated with a very poor prognosis (19).

A second report on bypass grafting for congestive failure was given in June, 1971, by Mundth et al (25) from the Massachusetts General Hospital, also describing experiences with 40 patients in whom congestive heart failure was the primary indication for operation. One-third of these had an associated ventricular aneurysm. There was a mortality rate of 22 percent, but the results obtained in surviving patients were better than those treated at New York University. Some improvement was obtained in 80 percent of those with angina as well as congestive failure, and about 70 percent of those who had congestive failure but no angina.

It should be emphasized that these data are preliminary, representing the first reports on this important question. Hopefully, better results will be obtained by other groups with wider experience. Certainly, triple bypass grafts should be employed in patients with diffuse disease, which was not done in the earlier patients operated upon at New York University. However, the absence of improvement in congestive failure with functioning bypass grafts is of considerable physiological as well as clinical significance. It suggests that failure is due to irreversible loss of functioning left ventricular muscle, as opposed to impaired function of viable muscle which may improve if ischemia is corrected with additional blood supply. Possibly bypass grafting may retard or prevent subsequent injury to the left ventricle, but improvement in impaired ventricular function apparently will be minimal in most patients.

These findings reiterate the crucial importance of ventriculography in estimating both the risk and potential benefits from bypass grafting. Bypass grafting is probably futile in patients with chronic severe congestive failure without angina, in whom there is diffuse severe impairment of ventricular contractility. Such patients usually have extensive widespread patchy destruction of left ventricular muscle. When advances in immunology make control of the rejection process more feasible, cardiac transplantation should be considered for this group of patients.

PREINFARCTION ANGINA

Preinfarction angina is a clinical syndrome characterized by a sudden increase in frequency and severity of angina, often combined with changes in the ST segments of the electrocardiogram. The diagnosis is not a precise one, but 40 to 50 percent of patients develop a classic myocardial infarction within the ensuing few days. With the favorable results following elective bypass grafting for angina, contrasted with the poor results in the presence of acute myocardial infarction (discussed in a subsequent section), the proper therapeutic approach for so-called preinfarction angina is assuming increasing clinical importance.

The pathologic finding that over 90 percent of patients with severe angina pectoris have obstruction of a major coronary artery, and the surgical finding that bypass grafts can be successfully inserted in over 90 percent of patients with occlusion of a main coronary artery, indicates that patients with a strong likelihood of developing a myocardial infarction constitute a true "emergency." A clinical program being employed with increasing frequency is the hospitalization of such patients under emergency circumstances with immediate coronary angiography, followed by transport of the patient from the angiographic laboratory directly to the operating room for bypass grafting. At New York University, several such patients have been hospitalized for emergency angiography. In some, interestingly enough, angiography did not disclose significant coronary artery disease and clearly an erroneous diagnosis had been made. In others with extensive disease demonstrated on angiography, immediate bypass grafting resulted in prompt relief of all symptoms and an uneventful recovery.

An extensive series was reported in April, 1971, by Lambert and associates (26), in which a total of 42 such patients had been so managed in an overall group of 250 patients undergoing bypass grafts. Uniformly good results were obtained, and similar less extensive experiences have been reported by other groups. In all likelihood, intense clinical studies will be pursued in the near future to determine the most reliable indicators of impending myocardial infarction with the possibility that immediate bypass grafting may prevent irreversible injury to the left ventricle.

ACUTE MYOCARDIAL INFARCTION AND ASSISTED CIRCULATION

A myocardial infarction which produces shock is a serious clinical problem, with a mortality rate in the range of 50 to 75 percent. The possible methods of surgical therapy under investigation in several centers include excision of the myocardial infarction, probably combined with bypass grafting. To date, mortality in most centers has remained above 50 percent; so, the procedures must be considered experimental at present. In the laboratory, experimental occlusion of a coronary artery produces irreversible ischemic injury of the myocardium within 45 to 60 min. Studies of changes in cardiac function following excision of the acute myocardial infarction are equivocal. Theoretically, additional blood supply from revascularization may prevent extension of the infarction. With the high mortality associated with such procedures, some type of temporary circulatory support is almost surely required if such operations are to become clinically feasible.

The most effective form of temporary circulatory support at the present time is intra-aortic balloon pumping for periods of one or more days. This has been experimentally evaluated in several centers (27). Circulatory improvement following intra-aortic balloon pumping, however, is often not sustained when the balloon pumping is stopped, probably because the basic cardiac injury remains unimproved. Hence, the combination of intra-aortic balloon pumping for temporary support, followed by angiography and bypass grafting, is undergoing clinical evaluation.

Austen and associates at the Massachusetts General Hospital have had the most extensive experience with this technique (28). As of September 1971 a total of 58 patients had been treated. Thirty of the 58 patients had intra-aortic balloon pumping alone, with only six long-term survivors. Although circulatory support was continued for several days in some patients, the longest that circulatory support was required in surviving patients was about 48 hr. Twelve patients undergoing open heart surgery for different conditions had circulatory failure after bypass and were supported with balloon pumping for a period of time, with four ultimate survivors. In 16 patients, temporary balloon pumping was employed, followed by coronary angiography and operation, usually excision of the myocardial infarction combined with bypass grafting. Mortality in this group of patients was near 50 percent. Undoubtedly this area of temporary assisted circulation combined with emergency angiography and operation will be an area of intensive clinical study in the next one to two years.

VENTRICULAR ANEURYSMS AND AKINETIC SCARS

Ventricular aneurysms develop following myocardial infarction in 10 to 20 percent of patients. Although the aneurysm may enlarge to a significant size, rupture is rare. Thrombi are regularly found in the lumen of such aneurysms, but for some fortunate but obscure reason, systemic embolism is infrequent. Large ventricular aneurysms apparently have a detrimental effect on myocardial function, probably by dissipating some of the contractile energy of the remaining functioning myocardium. In a classic review of 120 cases by Schlichter in 1954, 73 percent of the patients died within three years and 88 percent within five years (29). However, this dismal prognosis was only with aneurysms large enough to be detected clinically, for a study reported by Abrams in 1963 (30) of 65 aneurysms averaging 4 cm in diameter had produced no detectable complications during life. Most of these, however, had not been recognized clinically because of their small size and were only detected at autopsy.

As ventricular aneurysms are manifestations of coronary disease, an obvious corollary with evaluation of ventricular aneurysms is coronary angiography to determine the extent of coronary atherosclerosis in other vessels. Reconstruction of the coronary arteries supplying the area of the aneurysm may be of little benefit, as the regional muscle has been partly destroyed, but concomitant bypass grafts may be inserted to other obstructed coronary arteries at the time that the aneurysm is excised. In most patients, excision of a ventricular aneurysm is well tolerated. The most extensive series has been reported by the Cleveland Clinic group who have excised over 300 ventricular aneurysms with a mortality of near 10 percent in the last 100 procedures. Bypass grafts were employed as well in about one-half of the group (31).

Left ventricular scars, as opposed to a ventricular aneurysm, remain of uncertain clinical significance. Infarcted left ventricular muscle is gradually converted to a thin, parchment-like scar which may contract paradoxically with systole. Undoubtedly some contractile energy of the myocardium is wasted by paradoxical contractions in the scar, but the magnitude of the handicap probably depends upon the size of the scar as well as its mobility. Several studies have investigated the influence on myocardial function of excision of ventricular scars (32). Excision of large ventricular scars, greater than 5 cm, in patients with continued congestive failure after myocardial infarction, has been associated with a decrease in left ventricular end diastolic pressure and some clinical improvement (33). Excision of smaller scars, however, is seemingly not clinically significant.

In a separate category is the question of the value of excision of akinetic muscle, found to be viable but noncontractile on ventriculography. There are some hazards with attempted excision of akinetic areas, for the borders of akinetic zones often cannot be delineated precisely at operation, and areas of muscle which do not contract on ventriculography may conceivably improve in function following bypass grafting. For these reasons the procedure has not been employed extensively at New York University and remains on dubious clinical grounds at present (34).

ARTERIAL IMPLANTS

Although Vineberg performed his classic laboratory experiments a quarter-century ago, establishing that an internal mammary artery implanted into the myocardium remained patent in 70 to 80 percent of experiments, the clinical role of arterial implants for coronary artery disease remains uncertain (3). Arterial implants were used with considerable enthusiasm in the five-year period just preceding the development of bypass grafts in 1967 and 1968, but have been virtually abandoned by most centers since that time. The implants remain patent in 80 to 90 percent of patients but the increase in coronary blood flow through the implant has been disappointing in many patients. Sewell, previously one of the most ardent advocates of arterial implants, has virtually abandoned the procedure (35). At New York University, implants have been rarely performed in the past 18 months, usually in association with a bypass graft to another artery. By contrast, the Cleveland Clinic group and the Milwaukee group have continued to employ implants in 20 to 30 percent of patients operated upon (20, 21). No one questions that a bypass graft, when possible, is much more effective. The major question revolves about the effectiveness of bypass grafts to small (1 to 2 mm) diseased coronary vessels. With optical magnification a bypass graft can be attached to such an artery, with a subsequent flow rate in the range of 20 to 30 ml/min, but long-term patency rates are yet unknown. An alternate approach with a small diseased artery is to perform an adjacent arterial implant. Which of the two procedures is preferable is uncertain.

As mentioned earlier, over 90 percent of arterial implants remain patent. Patency seems most likely due to a to-and-fro movement of blood in the implant, with blood flowing away from the heart into the implant during systole, and from the implant toward the heart in diastole. This oscillating flow of blood is probably the major factor in determining patency. A second explanation for patency is the presence of myocardial sinusoids, providing some immediate runoff of blood from the implanted vessel and thereby avoiding stasis. Baird has recently published data (36) showing that initial flow in implants is nearly zero, and feels that the role of sinusoidal flow has been overemphasized.

The principal concern with implants, however, is the fact that the rate of blood flow may remain small, often only 5 to 10 ml/min, with no demonstrable influence on myocardial function. The ultimate rate of flow through an implant is determined principally by the collateral circulation which develops which, in turn, is probably influenced by the degree of ischemia in the muscle where the artery is implanted. Presumably the decreased arterial pressure in the ischemic vascular bed will enhance the development of collateral vessels from the implanted artery. Baird has defined additional factors which may influence the degree of collateral circulation developing from an implanted artery, but implantation into an ischemic zone of myocardium is probably the most significant factor.

In an occasional patient, dramatic clinical experiences indicate the beneficial effect of a previously performed implant. One graphic demonstration is the fact that the heart may continue to beat during a subsequent operation with the aorta occluded, all blood supply for the heart coming through the implant, for occlusion of the implant promptly produces cardiac arrest. A flowmeter in such instances may measure a rate of flow through the implant as high as 40 to 50 ml/min. Unfortunately, in other patients operated upon, a flow rate of only 5 to 10 ml/min is present (37). One of the best experimental studies demonstrating benefit from an implant was performed by Reis and associates in a series of chronic experiments over a period of one year, demonstrating a rather convincing protection by arterial implants from subsequent acute coronary occlusion (38).

Hence, at present, the principal question with arterial implants is how often they should be used as an adjunct to bypass grafting. A better definition of the factors which influence the development of collateral circulation around an implant would greatly facilitate this decision.

FUTURE CONSIDERATIONS

The feasibility of bypass grafting, and its widespread potential applicability to patients with coronary disease, is well established. It is theoretically possible to operate upon more than 90 percent of patients with symptomatic coronary artery disease, with immediate relief of angina in the majority, and an operative risk in the range of 5 to 10 percent. Several crucial questions require investigation in the next five years. The most important one is a better delineation of factors influencing patency of the bypass grafts, such as turbulence, comparison between autogenous arteries and veins, pericarditis, and probably other factors. A second major question is the influence of bypass grafts on the underlying disease, coronary atherosclerosis. Will atherosclerosis progressively involve the bypass grafts, progress at a similar unchecked rate in other coronary vessels, or perhaps be halted by appropriate dietary and other therapeutic measures?

Perhaps the most crucial question is the degree of protection provided by functioning bypass grafts from myocardial infarction and death. A controlled series, badly needed but perhaps clinically impossible in most areas, would be of great value in determining the difference in prognosis between patients with bypass grafts and those treated by nonoperative methods. However, the difficulty in establishing such a series, to be valid, would require coronary angiography in both operative and nonoperative patients, to be certain that the groups were indeed comparable. Otherwise, more seriously ill patients would be studied by angiography and operated upon, while those with less severe disease would probably be given nonoperative therapy.

The most impressive observation at the present time is the fact that infarction and death have been greatly reduced to date in patients with functioning grafts. If this favorable initial response is maintained for the next four to five years, with a dramatic reduction in death rate following bypass grafting, a much more widespread use of the procedure will undoubtedly be indicated.

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BIOCHEMICAL AND CELLULAR CHANGES IN CARDIAC HYPERTROPHY

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The clinical detection of cardiac hypertrophy is strong evidence for the presence of a significant pathological process and is often associated with myocardial decompensation. Nevertheless, cardiac hypertrophy in response to increased pressure or to volume overload imposed by disease should be considered an adaptive response that enables the heart to adjust its functional mass to work requirements. Cardiac muscle normally has a considerable capacity for the synthesis of proteins, nucleic acids and phospholipids. This capacity is necessary for maintenance of the structural integrity of the heart, since constituents such as myofibrillar proteins and mitochondria are normally in a state of continuous turnover. Cardiac synthetic processes are finely controlled by as yet poorly understood mechanisms so as to respond to physiological changes that alter the work load. When work demands are excessive, prolonged, or too rapidly applied, the synthetic and physiological compensations of the myocardium may be exceeded, or the organization of the muscle cell may be altered, with consequent diminished contractile function and failure.

During the last decade, considerable information has accumulated about some of the biochemical and cellular processes that accompany cardiac hypertrophy. A variety of experimental procedures has been developed which produce in animals a rapid or gradual increase in cardiac mass in response to increased work demand. These include constriction of the ascending or descending aorta or main pulmonary artery; renal or deoxycorticosteroid-induced systemic hypertension; damage to valves which results in aortic, pulmonic, or tricuspid insufficiency; chronic hypoxia, anemia, arteriovenous fistula, administration of thyroxine or isoproterenol, and severe prolonged exercise with a treadmill or an enforced swimming program. Cardiac enlargement secondary to genetically determined cardiomyopathy in the Syrian hamster has also been studied. Alteration of afterload in the per-

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fused rabbit or rat heart has been used to examine the more acute responses to increased work.

Cardiac growth stimulated by these procedures has been studied by a variety of biochemical, cytological and physiological approaches. These include the biochemical definition and analysis of the augmentation of protein and nucleic acid synthesis, and alterations in their rates of degradation; examination of the pattern of accumulation of various cell constituents; evaluation of changes in the metabolic pathways, in metabolite concentration, and in the capacity and efficiency of oxidative metabolism; and study of the properties of the muscle proteins, the sarcoplasmic reticulum and cell surface membranes. Histologic, cytologic, autoradiographic, and electron microscopic analyses have attempted to define the cardiac enlargement from the structural and organizational point of view, and physiologic examinations of the intact heart or of isolated papillary muscle have defined the functional changes that may be a consequence of increased cardiac muscle cell size. Our understanding of the processes involved in cardiac growth has also been greatly enhanced by the efforts of the developmental biologist studying skeletal and cardiac muscle cell differentiation in tissue culture and during embryonic life.

The characteristics of work-induced cardiac growth may differ greatly depending upon a number of experimental variables such as the severity, duration, and type (i.e., pressure or volume) of the overload, the rapidity with which it is imposed, the age of the animal and the condition of the myocardium. Many of the apparently conflicting reports in the literature can be resolved when these factors are taken into consideration. The process of cardiac growth may be conveniently separated into three stages: (*a*) *developing* cardiac hypertrophy, the period of active accumulation of myocardial components; (*b*) *compensated sustained* cardiac hypertrophy when a new steady-state with regard to synthetic and degradative rates has been attained; and (*c*) hypertrophy with *failure*, which may follow prolonged or severe increase in work load. The duration of the three stages, and the progression from one to another, depends on the magnitude and nature of the work load. If an excessive load is imposed too rapidly, immediate failure may result. Cardiac growth induced in the fetal or neonatal period may differ fundamentally from that in the adult, in that the element of hyperplasia may be present, as will be discussed below in more detail.

The purpose of this review is to summarize some of the recent advances in this field. It must be emphasized, however, that despite the considerable progress made by the multidisciplinary approach, many of the most fundamental questions about cardiac hypertrophy and, indeed about growth of any differentiated organ, have not yet been answered. Still unknown is the mechanism by which a physiological stimulus such as increased aortic pressure or cardiac output results in the extremely complex but organized intracellular response culminating in the accumulation of new, functionally effective, structural units. Equally obscure is the reason for the apparent loss, by the adult or postneonatal cardiac muscle cell, of its capacity for mitosis, so that cell enlargement rather than proliferation occurs except in exceptional cases to be described below. This limitation in cardiac cell division is possibly also the basis of the inability of postnatal cardiac muscle to regenerate after injury (1, 2), contrasting with the considerable capacity of skeletal muscle for repair and regeneration (3, 4). Also still escaping adequate explanation is the apparent decline in contractile efficiency of isolated hypertrophied cardiac muscle, and the cause for development of cardiac failure in hearts subjected to chronic or excessive hypertrophy.

Other reviews (5–9) and proceedings of symposia (10, 11) on aspects of cardiac hypertrophy have been published.

CARDIAC ENLARGEMENT: HYPERTROPHY OR HYPERPLASIA?

According to classical pathological teaching (12, 13) the adult heart enlarges exclusively by increasing the size of existing cells (i.e., hypertrophy) and not by increasing cell number (hyperplasia). The heart thus differs from organs such as the liver (14, 15) and kidney (14, 16, 17) in which cell division is a prominent component of growth. This conclusion is based on histological observations that the cardiac muscle cell increases in diameter, length, and volume during growth but that the number of cells do not appear to increase (18–21). Mitoses are never seen in the adult muscle cell. Recent biochemical and quantitative cytological observations have modified this conclusion to some extent. Cardiac enlargement produced by a variety of experimental procedures invariably leads to an increase in DNA content and mitotic index, suggesting cardiac cell division (22–30). Radioautographic studies with ^{3}H-thymidine, however, have shown that the DNA synthesis is confined to the connective tissue cells (27, 28, 31–33). The increased mitotic index (29) reflects division of connective tissue cells only. Furthermore, differential counts of connective tissue nuclei and muscle cell nuclei show that while the former do, the latter do not increase in number during growth (27, 30, 33). Thus, cardiac enlargement in the experimental animal appears to consist of hypertrophy of muscle cells and hyperplasia of connective tissue cells.

Cardiac enlargement produced in neonatal animals by nutritional anemia (34, 35) or by aortic constriction (36) may differ from that in adult heart in that a hyperplastic component may be present. Muscle cells display considerable mitotic activity in the neonatal heart and this activity declines rapidly after birth (37–43). The number of altered mitoses, possibly reflecting block in the process particularly in prophase and telophase, increases abruptly during the first few days after birth, and pairs of nuclei produced by karyokinesis without cytokinesis accumulate during the second postnatal week (44). Cell divisions are no longer seen after the third week of life in the rat; the number of cardiac muscle cells is fixed thereafter. It is likely therefore that cardiac enlargement stimulated during the fetal or neonatal period may differ fundamentally from that appearing in the adult heart in that true muscle hyperplasia may participate. The implications for congenital heart disease are obvious.

An increasing degree of polyploidy of cardiac muscle cell nuclei contributes only slightly (29), if at all (30, 45), to the increased myocardial DNA seen in experimental cardiac enlargement in rats, but is of considerably greater magnitude in human pathological hypertrophy (46–48). This increase in the number of chromosomal sets per nucleus probably accounts for the incorporation of ^{3}H-thymidine by an occasional muscle cell nucleus (33). In normal rat heart, only 2 percent of cardiac muscle cell nuclei are polyploid so that the twofold increase in polyploid frequency noted after aortic constriction is quantitatively inconsequential (29). In the adult human heart, however, more than 80 percent of muscle cell nuclei are normally tetraploid and severe cardiac enlargement leads to a marked shift to octaploid nuclei and nuclei of even higher ploidy (16c and 32c) (46, 48). The cause and significance of polyploidy is unclear, but it may explain in part Linzbach's careful quantitative findings that after a critical myocardial increment in mass of the left ventricle is attained, there is an increase in the number of myocardial cells (49–51), an observation which has recently been made for right ventricular enlargement as well (52). Since mitotic activity has not been observed in the adult heart, it has been suggested that the cell divides by a process of longitudinal splitting (49–51), which includes division of the polyploid nucleus.

The basis for the loss of mitotic activity by the adult cardiac muscle cell is unknown. During differentiation, cardiac muscle cells remain mononucleated while skeletal muscle cells fuse to form multinucleated myotubes. Whereas in embryonic skeletal muscle the onset of synthesis of specific contractile proteins coincides with the cessation of mitotic activity (53–55), embryonic cardiac muscle cells in tissue culture retain mitotic activity even after the appearance of myofibrillar proteins (56, 57). There is no evidence for the presence of undifferentiated myoblasts or "satellite cells" in adult heart (57–59), as appears to be the case in skeletal muscle (58, 60–64). The loss of mitotic capacity and regenerative ability of the adult heart (1, 2) is probably an important factor limiting the magnitude of cardiac growth.

Although the mitochondrial DNA of myocardial cells (65, 66) probably also increases in cardiac enlargement (67), it is present in relatively small amounts (1–2 percent of total DNA), so that it can contribute very little to the observed elevations in total cellular cardiac DNA in hypertrophy.

PROTEIN AND RNA SYNTHESIS

The rate of protein synthesis in normal cardiac muscle, judged by ribosomal RNA content and by amino acid incorporation into protein, is relatively slow compared to that of organs such as liver and bone marrow which manufacture extracellular and intracellular protein for export (68). During steady-state conditions the heart needs only to replace structural constituents, which are normally in a state of continual turnover. For example, two of the major protein constituents of rat heart muscle, myofibrillar and mitochondrial proteins, turn over with half-lives of 10–12 (69, 70) and 5–6 days (66, 71–73), respectively, in the rat. After stimulation of growth by aortic constriction, cardiac mass and protein content increase by 30–50 percent in 48 hr (68, 74), representing the synthesis of 1 mg protein/(hr/g tissue), which may be compared to reticulocytes making hemoglobin at a rate of 6 mg/hr/g of tissue (68).

The increased rate of protein synthesis during developing cardiac hypertrophy appears to be related primarily to an increase in the number of myocardial ribosomes. Increased RNA concentration per gram of heart as well as increased total cardiac RNA content reflecting the increased number of ribosomes, have been observed in cardiac growth induced by a variety of experimental procedures (7, 22–26, 35, 75–80). A 3- to 4-fold enhancement in the incorporation of ^{32}P and ^{3}H-uridine into ribosomal and tRNA, corrected for changes in precursor pool size and specific activity, reflects the markedly increased rate of RNA synthesis (79, 81). Of interest is the expansion of the uridylic acid pool (81), possibly the result of the observed increase in uridylate kinase activity (82). Analysis of pulse-labeled, newly synthesized RNA by sucrose density gradient centrifugation reveals equal incorporation of label into ribosomal RNA and tRNA with no evidence for the presence of high specific activity messenger RNA (81, 83). Also there was no demonstrable change in polysomal profiles or in the response of isolated microsomes or ribosomes to polyuridylic acid in cardiac hypertrophy (68, 78, 84). All these observations suggest that a balanced increase in all RNA components and an increase in ribosomal number are the major determinants of the increased protein synthesis.

In more acute studies of the perfused heart, Schreiber et al have presented evidence suggesting selective messenger RNA synthesis (85). Although the syntheses of messenger RNA and ribosomal RNA appear to be parallel in less acutely induced growth, alteration in the types of messenger RNA made are suggested by Florini & Dankberg's findings that two days after aortic constriction there were substantial changes in the labeling pattern of myocardial soluble proteins separated by isoelectric focusing (86). The stimulation of ribosomal RNA synthesis is reflected morphologically by enlargement of the nucleoli (7, 87).

An early biochemical change observed in developing cardiac hypertrophy is that in nuclei isolated from heart there is an increase in the activity of RNA polymerase, the enzyme responsible for RNA synthesis. This change has been observed within 12–24 hr after aortic constriction in the rat (75) and within 20–60 min following elevation of aortic pressure in the perfused rat heart (88). Inhibition of protein synthesis by puromycin during the first 4 hr of aortic constriction prevents the increment in incorporation of ^{32}P into RNA observed at this time (89), suggesting the participation of newly synthesized protein in the early activation of RNA transcription. To date there is no evidence for translational control of protein synthetic activity such as appears to be operative in the stimulation of skeletal muscle protein synthesis by insulin (90, 90a).

Mitochondria isolated from enlarging rat heart also show increased incorporation of amino acids into protein (91), in keeping with the substantial mitochondrial accumulation during early hypertrophy.

In the sustained phase of cardiac hypertrophy, with and without failure, total RNA and DNA content of the heart falls from the peak levels observed in developing hypertrophy but still remains above control levels (24, 29, 79, 80). Heart weight and protein content remain elevated, and the concentrations of both RNA and DNA per gram of heart tissue fall below normal (22–26, 29, 79, 80). The rate of protein synthesis, as reflected by the level of incorporation of labeled amino acids, also declines toward normal (27, 92). Additional detailed studies of protein synthesis and degradation during chronic hypertrophy with and without failure are needed.

CHANGES IN TURNOVER OF MYOCARDIAL COMPONENTS

Although the cardiac muscle cell may have a life as long as that of the entire organ, individual components within the cell turn over quite rapidly. For example, in rat heart the mitochondrial inner membrane and its cytochromes turn over with a half-life of 5–6 days (66, 71–73), and the myofibrillar proteins, myosin, actin, tropomyosin, and troponin with a common apparent half-life of 10–12 days (69, 70). Earlier studies had indicated a definitive life span for skeletal muscle myosin (93) analogous to that of hemoglobin, rather than random destruction characteristic of most other intracellular proteins. Morkin's autoradiographic studies suggest that newly formed myofibrils are assembled on the surface of the muscle fiber in the rat diaphragm (94). Relative stability of surface myofibrils and a more rapid destruction at the center of the fiber would explain the kinetics of decay of labeled proteins. Efficient re-utilization of labeled amino acid released after destruction of various intracellular proteins, however, makes interpretation of labeling curves difficult. Studies attempting to minimize re-utilization indicate more rapid turnover rates of muscle proteins, and suggest random destruction (95–97). The initial half-life of RNA in newborn and adult mouse heart is 5 days (99), similar to that found for liver ribosomal RNA (98). Morkin's preliminary findings suggest a decrease in RNA degradation during developing hypertrophy (100).

The normal continuous process of destruction and replacement of myocardial components enables the heart to adapt more effectively to an increase or decrease in workload. The steady-state level of any cellular constituent is the resultant of the rates of its synthesis and destruction (101). In cardiac hypertrophy (70, 102, 103), as in skeletal muscle hypertrophy (104), a decrease in the normal rate of destruction of myocardial constituents such as myosin and mitochondria contributes to the accumulation during the growth phase. After aortic constriction in the rat, the rates of degradation of myosin (70, 102) and of cytochrome *c* (70, 103) decrease considerably during the early rapid phase of growth. Similarly, a decreased degradation of cardiac mitochondrial protein contributes greatly to mitochondrial accumulation after thyroid hormone administration to hypothyroid or euthyroid rats (105, 106). In the rabbit, however, the decreased rate of myosin destruction was not observed after aortic constriction (107). It is probable that the rate of myofibrillar protein degradation increases during regression of cardiac hypertrophy as has been reported to be the case in skeletal muscle (96). Although a procedure for removal of the constricting aortic band has been described (108), changes in protein turnover have not yet been studied in this condition. The mechanisms controlling the rate of intracellular turnover are still almost completely unknown although lysosomal degradative enzymes presumably participate (109). Studies in bacteria suggest that the level of aminoacylation of tRNA may be involved in the control of protein degradation (110).

Meerson has suggested that during sustained hypertrophy wear and tear may lead to an increased breakdown of myocardial proteins and may contribute to the appearance of failure (27). No evidence for increased destruction of total cardiac proteins or of myosin, however, has been observed during sustained hypertrophy (102, 111). Further work with individual proteins is necessary to determine whether protein turnover is altered in chronic hypertrophy or in congestive failure. The possibility that transient protein degradation is the signal that initiates the growth process will be discussed below.

PATTERN OF ACCUMULATION OF MYOCARDIAL COMPONENTS IN CARDIAC HYPERTROPHY

In the developing and in the compensated sustained stages of cardiac hypertrophy a relatively global pattern of accumulation of muscle cell elements has usually been observed, although individual examples of preferential synthesis of myocardial constituents have been described. Increases in respiratory enzyme activities or mitochondrial protein concentrations have been reported in the very early stages of the development of cardiac hypertrophy in response to pressure overload (7, 22, 73, 103), in aortic insufficiency (80), after exercise (112), and after thyroid hormone administration (73, 106, 113) but no changes in respiratory enzyme concentrations were noted 3 to 10 days after construction of an A-V fistula by Dart & Holloszy (114) or after exercise by Oscai et al (115). Electron microscopic measurement of the mitochondrial/myofibrillar ratio, and quantitative measurement of the volume of cell constituents at various stages of hypertrophy suggest a relatively increased mitochondrial volume at the earliest time periods (116, 117), a proportional accumulation of subcellular constituents during the later developing and sustained stages of hypertrophy (116, 118, 119), but a relative decrease in mitochondrial and increase in myofibrillar volume later in hypertrophy (116, 119–121). The T-tubular and sacrotubular membrane systems appear to increase extensively so as to keep constant both the total cell surface to volume ratio, and the ratio of sarcotubular membrane area/myofibrillar volume, at least in the case of thyroid-induced cardiac enlargement (122). Additional studies of this type in different models of hypertrophy, and especially in the decompensated stages of cardiac enlargement, will be of great interest.

The connective tissue cell proliferation described above is accompanied by an increased collagen accumulation in cardiac growth secondary to increased pressure work (33, 80, 92, 123–126). The incorporation of ^{3}H-thymidine into nuclear DNA of connective tissue cells is elevated two days after aortic constriction, and reaches a peak four to eight times above control values about a week after surgery (92, 125). Synthesis of collagen proceeds in two phases. First, there is a substantial initial burst of labeling which peaks as early as two days after surgery, and thus precedes the proliferation of connective tissue cells by several days, suggesting activation of collagen synthesis in pre-existing cells. This is followed by a lower level of sustained but still enhanced collagen synthesis for as long as a month after aortic constriction. The concentration of hydroxyproline per gram of heart increases in most types of cardiac growth (92, 125), but not in hypertrophy produced by thyroxine or sideropenic anemia (123). In the isolated perfused heart, increasing aortic pressure results in a consistently enhanced incorporation into myosin but not into collagen (127). It is still unclear whether the increase in collagen is an essential part of the growth process and is involved in the proliferation of new vascular, sarcotubular, and other connective tissue structural elements, or whether it is in large part a pathological fibrotic repair response associated with the minor trauma consequent to the greatly increased workload. The role of the increased collagen content in the altered mechanical properties displayed by hypertrophied papillary or trabecular muscle is not clear.

The delivery of oxygen to the center of the enlarged muscle cell may be impaired in hypertrophy. The diffusion of oxygen and nutrients may be further compromised by a relatively less-developed capillary bed. During normal growth of the rabbit (128) or human (49, 129) heart, capillary growth occurs in proportion to the increase in muscle cell size, resulting in a constant density of capillaries per square millimeter of muscle tissue. In cardiac enlargement produced in the newborn rat by anemia (34, 36, 130) or aortic constriction (36, 128, 130), both capillaries and muscle cells proliferate with no change in capillary density per unit area of tissue. In contrast, a diminished capillary density, with an increased calculated diffusion distance was observed in cardiac enlargement in older animals (36, 130). In hypertrophied human heart, Roberts & Wearn (129) found no evidence of capillary proliferation, so that capillary density decreased. Linzbach (49, 131) and Astorri et al (52), however, observed an absolute increase in capillary number in greatly enlarged hearts. The latter group noted a decreased capillary density however, indicating that cell enlargement had outstripped capillary proliferation.

Some of the differences in the patterns of accumulation of myocardial components observed by different laboratories may be due to the variations in the methods used to stimulate growth. The abrupt establishment of aortic stenosis or aortic insufficiency, used by most investigators, is very different from the gradual development usually observed in disease, and may contain elements of a destructive nature that are not an essential part of the growth stimulus. Scattered necroses and myofibrillar changes similar to those seen in hypoxia, have been observed by electron microscopy (132) within the first few days after production of aortic constriction. Statistical analysis of the spatial distribution of dividing connective tissue nuclei during this period indicates a changed, nonrandom distribution, compatible with the possibility of focal necrosis (33). In developing hypertrophy, myofibrillar changes, consisting of distortion of the I-band and Z-line, have been described (133, 134). These may represent the formation and insertion of new sarcomeres into the myofibrils (133, 134), or true lesions of the contractile proteins (134). Swelling of mitochondria has also been noted in the early developing period of hypertrophy (120). In the period of established hypertrophy there are no striking ultrastructural changes other than a reduced ratio of mitochondrial to myofibrillar volume (116, 119–121). In acute and in chronic failure secondary to aortic constriction, mitochondrial swelling, changes in contractile structure, splitting of myofibrils, and disintegration of the Z-band have been observed (120, 135), but are not always correlated with the degree of failure (134).

CHANGES IN THE PROPERTIES OF MYOCARDIAL COMPONENTS

Myofibrillar Proteins

The physical and enzymatic properties of myofibrillar proteins have been studied extensively to try to explain the diminished contractile efficiency in long-standing hypertrophy. No alterations have been detected in the physical properties of cardiac myosin isolated from hypertrophied failed hearts (136, 137), discounting earlier reports (138). To date no evidence has been presented for changes in the recently described light chains of myosin (139) which are presumed to be involved in the control of ATPase activity (140).

Myofibrillar and myosin ATPase activities are related to energy utilization for muscle contraction. Decreased ATPase activity may indicate a change in one of the myofibrillar proteins, or in a regulatory factor, that might be responsible for decreased contractility. Studies of changes in cardiac myofibrillar and myosin ATPase in hypertrophy and failure, however, have been contradictory and must be interpreted with caution (see Katz for critical review, 141). Some of the difficulties arise from contamination of conventionally prepared cardiac myofibrils by mitochondria, membranes, nuclei, and ribosomes (142). Most authors agree that myofibrillar ATPase is not altered in compensated hypertrophy (80, 143–145), and is decreased in chronic hypertrophy (145) and heart failure (143, 144, 146, 147). No differences were found, however, with glycerinated muscle fibers (148) and when ATPase of the failing heart was assayed histochemically (149), although the latter measurement is semiquantitative at best. Several groups found actomyosin ATPase to be depressed in chronic hypertrophy (80, 147a, 150). However, in an excellent recent study, ATPase of well-characterized actomyosin was normal in both chronic hypertrophy and heart failure even though myofibrillar ATPase was decreased (145). Conflicting results have also been obtained in studies of actin-myosin interaction (141). Increased myosin ATPase activity both in hypertrophy (7) and in heart failure (151) has also been reported but requires confirmation. Further investigations with purified protein preparations in defined physiological states are necessary to evaluate the possible contribution of depressed myofibrillar ATPase to decreased contractility in cardiac hypertrophy.

MITOCHONDRIAL FUNCTION

Most studies of this problem have found normal oxidative metabolism in compensated hypertrophied heart. Mitochondria isolated from these hearts are usually reported to have normal P:O ratios and normal respiratory control (152, 153). Tissue ATP and creatine phosphate levels have been found to be normal in some studies (154, 155), with creatine phosphate depressed in others (156, 157). There is controversy about the function of mitochondria isolated from decompensated hypertrophied hearts. Although some groups have observed no abnormalities even in the presence of failure (152, 153, 158), decreased P:O ratios (159, 160) and a depression in respiratory control ratio has been reported in failure (161, 162), especially when special precautions are taken in the preparation of the mitochondria (162). Whether these abnormalities contribute to the decline in myocardial function has yet to be established.

SARCOPLASMIC AND CELL SURFACE MEMBRANE

It is now widely held that the basic mechanisms of excitation-contraction coupling and relaxation in cardiac muscle (163–167) are similar to those established for skeletal muscle (168–172), and involve the release and subsequent uptake of calcium by the sarcoplasmic reticulum membrane. It has been postulated, but not established, that mitochondrial calcium accumulation may also participate in the relaxation process (173–177). To try to account for the depressed myocardial contractility observed in hypertrophy and failure, the properties of membrane fractions isolated from hypertrophied and failing hearts have been studied with respect to calcium binding and uptake, and calcium-dependent ATPase activity. Since all preparations of cardiac sacroplasmic reticulum are cross-contaminated with other cellular membranes, sodium azide (163, 164), which presumably selectively inhibits mitochondrial calcium uptake, has been used to obtain greater specificity.

Abnormalities in calcium binding and uptake have been observed in spontaneously failed heart-lung preparations (178) and after coronary occlusion (179) in dogs, in right ventricular failure secondary to pulmonary artery constriction in calves (180), in failed human hearts (181), and in the hypertrophy and congestive failure of genetic myopathy in the Syrian hamster (182–184). With either the murexide (181, 182) or the $^{45}Ca^{2+}$ millipore technique (182, 184), the rate and extent of calcium *binding* (in the absence of oxalate) per milligram of isolated sarcoplasmic reticulum was reduced in failed hearts of hamsters (182, 184) and humans (181). In myopathic hamsters, the rate of Ca^{2+} *uptake* (in the presence of oxalate) was found to be normal (184) or reduced (182, 183), and the maximal amount of Ca^{2+} bound per milligram of sarcoplasmic reticulum normal (183) or reduced (182). Although Gertz et al (183) observed a normal maximal uptake per milligram of membrane, total cardiac uptake was diminished, suggesting a decrease in total amount of membrane responsible for Ca^{2+} uptake. A depression of the rate and extent of mitochondrial Ca^{2+} binding in failure in the myopathic hamster has also been reported (184). Decreased rates of Ca^{2+} uptake were observed in congestive failure secondary to pressure-induced hypertrophy (180), and in failing heart-lung preparations (178).

Diminished sarcotubular reticulum ATPase has been described in failure secondary to pulmonary artery constriction in the calf (180) and dog (185), but the Na^+, K^+ dependent sarcolemmal ATPase was not changed (185). No change in total calcium content of failed and normal hearts has been found (186). It is apparent that the amount, or activity of the calcium-accumulating system in heart may be altered in congestive failure, but whether this is a primary or secondary effect is yet to be established. Quantitative measurement of the sarcoplasmic reticulum by electron microscopic stereological techniques (187, 188) in hypertrophy and failure will be of great interest.

STIMULUS FOR HYPERTROPHY

Although considerable information has accumulated describing the biochemical and cytological changes in the hypertrophied cardiac muscle cell, there is little known about the mechanism of initiation of the integrated growth response by the physiological stimulus.

Among the hypotheses suggested are (*a*) that increased work demands lead to a local tissue hypoxia (5) or depletion of energy stores (6) with accumulation of metabolites which in turn induce or derepress the synthetic process; (*b*) that increased wall tension or hypoxia results in a "wear and tear effect," leading to the breakdown of macromolecules with the release of substances that stimulate growth (7); (*c*) that stretch of the muscle cell secondary to enhanced preload or afterload results in the augmented synthesis; (*d*) that humoral or hormonal factors participate in the hypertrophy. The hypoxia hypothesis is based on the observation that ischemia, and presumably chronic hypoxia secondary to coronary artery disease, are often associated with hypertrophy (189). Furthermore, all the various types of increased workload increase the oxygen demands of the muscle. Hypoxia has been shown to promote the formation of new capillaries in heart muscle (190), to stimulate growth in tissue cultures (191), and to stimulate RNA synthesis in the perfused heart (192). Some evidence has been presented for very early preferential synthesis of mitochondria in cardiac growth which lends some support to this hypothesis (80, 103, 112). A shift of the lactic dehydrogenase isozyme pattern to one closer to that seen in embryonic or anaerobic tissue was observed in dogs with right ventricular hypertrophy by Fox & Reed (193), but no such change was observed in rats with left ventricular hypertrophy by Bing et al (126). If operative, the hypoxic stimulus must work over a narrow range of oxygen tension since anoxia and diminution in energy stores have been shown to inhibit protein synthesis in the isolated perfused heart (194) and in isolated atria (195).

No evidence for the participation of macromolecular breakdown in initiation of the growth process has been forthcoming. Although a transient destruction of mitochondrial components has been observed immediately after aortic constriction the effect was inconsistent, and may have been the result of hypoxia during the operative procedure (70, 103). Hypoxia has been shown to result in mitochondrial destruction in the heart (196).

Several examples of stretch resulting in enhanced muscle growth have been described. Insertion of a rubber ball into the uterine cavity results in increased incorporation or precursors into RNA and protein, and in muscle growth (197). Similarly, denervation of a rat hemidiaphragm results initially in its hypertrophy, presumably because it is passively stretched by the contralateral hemidiaphragm (198, 199). Elimination of the stretch stimulus by denervation of both diaphragms, or by costotomy prevents the hypertrophy (198, 200). Electron microscopy has shown that myocardial nuclei undergo considerable change in their conformation during the contraction-relaxation cycle (201). Such deformations or alterations in the spatial relations of myocardial subcellular components, could conceivably activate biochemical processes and initiate growth.

No humoral agent responsible for cardiac growth has yet been isolated, although it is established that adrenocortical, thyroid, and growth hormone enhance pressure or isoproterenol-induced hypertrophy, perhaps by their secondary hemodynamic effects. Their presence does not appear to be essential to cardiac (202–204) or skeletal muscle (205) hypertrophy.

Possible steps involved in translating the physiological signal into cell growth are increased transport of precursors such as amino acids and nucleosides into the cell, and the participation of the adenyl cyclase system. Increased rate of amino acid transport (206–208) and increased size (206) of the amino acid and uridylate pools (81) have been observed during enhanced muscle growth, and incorporation of amino acids into protein has been correlated with tissue levels of amino acids (206). Furthermore, passive stretching of papillary muscle in vitro has been shown to increase penetration of amino acids into the cell (209).

Elevated cardiac adenyl cyclase activity has recently been demonstrated

15 min after increasing the aortic pressure in the perfused rat heart (210). Elevations are particularly marked in hypertrophy resulting from genetic cardiomyopathy in the Syrian hamster (211). Although these observations may only represent effects secondary to catecholamine release, the possible participation of the adenyl cyclase system in the process of cardiac growth must be considered in the light of recent demonstrations that cyclic AMP may be a regulator in RNA transcription. Cyclic AMP has been shown to be involved in hydrocortisone-induced enzyme induction in rat liver (212, 213), and in glucose repression of degradative enzyme synthesis in *Escherichia coli* (214–218) and mitochondrial development in yeast (219). A direct stimulating effect of cyclic AMP on nuclear RNA polymerase has been observed in rat liver (220) and rat heart (A. Cutilletta, unpublished observation). The early stimulation of nuclear RNA polymerase activity in cardiac hypertrophy (74, 88) and the increased template activity for RNA polymerase of chromatin isolated from nuclei of hypertrophied heart (86), together with the inhibition of the development of hypertrophy by actinomycin D (203, 221, 222), indicate that regulation on the transcriptional level is probably fundamental to the initiation of cardiac enlargement.

PHYSIOLOGICAL CONSEQUENCE OF CARDIAC HYPERTROPHY

The development of small-to-moderate degrees of cardiac hypertrophy seems to be an effective mechanism by which the heart can compensate for increased functional requirements. Excessive and prolonged work demands, however, may result in failure. Normal or enhanced hemodynamic function was observed in animals with hypertrophied but nonfailing hearts (223–227). Careful study of muscle mechanics of papillary or trabecular muscle hypertrophied in response to pressure overload, however, suggest a diminished contractility per unit muscle even in apparently compensated hearts (126, 228). Right or left ventricular pressure-induced hypertrophy results in decreased maximal velocity of shortening, and maximal rate of tension development, but peak isometric tension development corrected for cross-sectional diameter is unchanged. The observations may represent intrinsic alterations in contractility, changes in the time course or intensity of the active state and/or alterations in the parallel elastic components of the muscle. The latter must be considered especially in view of the increased collagen content of hypertrophied muscle. The abnormalities in mechanics were present to a greater extent in muscles isolated from decompensated hearts (228). Diminished maximal velocity of shortening, and tetanic tension corrected for cross-sectional area (Po), have also been observed in skeletal muscle hypertrophy (229, 230) where evaluation of contractile element properties is less difficult. In contrast to pressure-induced hypertrophy, no abnormalities in the force-velocity relationship was observed in dog hearts enlarged in response to volume overload (231, 232). Isolated perfused hearts from animals conditioned by a chronic exercise program exhibited increased metabolic and dynamic performance in the steady-state, and increased aerobic and mechanical reserve when stressed (233). Thus, there may be differences between volume and pressure overload-induced cardiac enlargement. The apparently normal or even improved contractile function of the conditioned heart may be due either to the smaller degree of cardiac enlargement obtained with exercise, or to a fundamental difference in the response of the heart to the more "physiological" exercise stimulus.

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CHEMICAL MEDIATION OF HORMONE ACTION 7041

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INTRODUCTION

The multiplicity and complexity of effects produced by hormones do not readily permit the development of mechanistic hypotheses. In evaluating the consequences of a hormone stimulus, temporal sequence remains the most reliable indicator of causal effect. Thus, as with Occam's razor, everything is explained in terms of a single cause with emphasis on the initiating event.

For all classes of hormones, there is a first encounter with a receptor molecule, in the plasma membrane or cytoplasm. Identification of the initial locus of interaction between hormone and cell is indispensable for the proper understanding of derivative molecular sequences.

Because of our predilection and the availability of detailed information, we emphasize the molecular interactions initiated by the adenyl cyclase hormones. Insulin, thyroxin, and steroid hormones are also discussed. The classical theory that hormones initially and derivatively work by effecting enzymic activity has been confirmed in a mechanistic sense for the cyclase system. In contrast, steroid hormones affect the levels of enzyme synthesis rather than the activity of preformed enzyme molecules. Because of the explosive growth in molecular endocrinology, it is inevitable that the material described will soon be supplanted by new details and finer insights.

ADENYL CYCLASE

INTRODUCTION

Adenyl cyclase is a membrane-located enzyme which regulates specialized cell functions by synthesizing cyclic 3′5′-AMP (cAMP) exclusively in response to selective hormonal stimulation. Examples of cAMP-dependent functions include substrate mobilization (1, 2), melanosome dispersion (3, 4), hormone release (5–8), the regulation of urinary osmolality (9), and sensory (10) and neural excitation (11). In each tissue, adenyl cyclase exhibits unique hormone-binding characteristics and specificity (12–14), suggesting discrete regulatory subunits which transduce hormonal signals into changes in adenyl cyclase activity. (For additional cyclase-related functions, see reference 30.)

HORMONE BINDING AND ACTIVATION

Evidence for the binding of hormones to membrane receptors includes the observation that activation of liver cyclase by glucagon in vitro is not reversed by extensive washing of the particulate enzyme. This suggests firm binding between glucagon and its receptor (15, 16). Thyroid-stimulating hormone (TSH) activates the adenyl cyclase of thyroid cell suspensions. Although this effect is not reversed by washing with buffer, it is reversed by rinsing the cells with TSH antibody (17) or trypsin (18). This illustrates not only firm binding, but also that hormone activation occurs at the surface of the cell (since neither trypsin nor TSH-antibody traverse the cell membrane). After more prolonged incubation with TSH, binding was no longer reversible by antibody (17). The glucagon activation of adenyl cyclase in purified hepatic membrane particles is proportional to labeling of these particles with I^{131}-glucagon (19). These experiments again suggest that glucagon binding is a necessary component of the activation of hepatic cyclase.

Labeled ACTH binds to tissue cultured adrenal cells, but in the absence of calcium activation of cyclase does not accompany binding (13). Activation of adrenal cell cyclase by ACTH is proportional to the addition of millimolar quantities of calcium. These studies demonstrate that binding of the peptide is necessary but not invariably sufficient for the activation of adenyl cyclase. Calcium also has a role in the activation of toad bladder adenyl cyclase by vasopressin (20).

Guanosine triphosphate alters the binding and release kinetics between glucagon and its hepatic membrane receptors and stimulates formation of cAMP in response to glucagon. Guanosine diphosphate is equally effective while uridine, cytidine, and adenosine triphosphate work only at much higher concentrations. Experiments with nucleotide imidodiphosphates indicate that this effect does not depend on a phosphorylation reaction (21).

The importance of amino acid sequence and content of glucagon for the activation of liver cyclase has also been studied. Deletion of a dipeptide at the C-terminal portion of glucagon (asparaginyl-threonine) reduces activation, and the synthetic glucagon sequence 1–23 (with 24–29 deleted) is completely inactive as in the fragment 18–29 (14). Removal of the histidine at the C-terminal portion of glucagon results in complete loss of activity (22, 23). The presence of selective blocking agents on either the N-terminal or epsilon amino groups prevents activation of cyclase (23). These studies confirm the suggestion that those portions of peptides which contain basic amino acids are especially important in binding to receptors (24).

Interesting structure-activity relationships have been described for oxytocin. Oxytocin analogues (N-acetyl, O-acetyl and N-carbamyl, O-carbamyl oxytocins) have been prepared which appear to retain affinity for their uterine receptors, but which interfere with the function of native oxytocin and do not by themselves induce uterine contractions (25).

Although earlier studies of structure-activity relationships for ACTH and melanocyte-stimulating hormone (MSH) depended on bioassays rather than cyclase activation, these studies demonstrate the amino acid sequence requirements for binding and activation of the respective cyclase systems (26, 27). The similarities in amino acid sequence between MSH and ACTH indicate similarities between the hormone receptor moieties for cyclase in adrenal cells and melanocytes (3, 4, 28).

The logistics of binding to and activation of a distant tissue by a peptide (e.g., vasopressin in the kidney) are complicated by dilution and nonspecific inactivation in the general circulation. Here, amounts of peptide needed and the affinity requirements for binding would seem to be much greater than in those cases (e.g., hypothalamic-releasing peptides in the pituitary and alpha cell glucagon in the liver) in which anatomical arrangements provide privileged access and markedly reduce the distance traveled by the hormone, and the accompanying dilution.

Little is known about removal of peptide hormones or deactivation of adenyl cyclase. Racemization of MSH with 0.1 *M* sodium hydroxide produces a peptide which darkens frog skin melanocytes but whose effects are no longer reversible by rinsing with frog ringers (29). This could indicate that stereo-specific enzyme reactions are necessary for peptide removal. Alternatively, racemization could increase the affinity of the peptide for its receptor. The high affinity of ACTH for its receptor (30) might suggest a need for enzymic removal of peptide hormones. However, at 24°C spontaneous dissociation of ACTH from its receptors occurs with a half-time of about 15 min (private communication, J. Roth). Cyclase in membrane particles prepared from livers of animals given intraportal glucagon shows no persistence of activation while remaining sensitive to glucagon added in vitro. This contrasts with the persistence of glucagon activation in vitro (31). Studies with insulin analogues and purified liver membranes indicate that the efficiency of binding to receptors correlates perfectly with the production of insulin effects, while rates of degradation by the membranes are independent of function. These studies suggest that insulin inactivation occurs at sites which are independent of the functional sites (J. Roth, personal communication). The characteristics of peptide removal or deactivation are an important aspect of the transactions between adenyl cyclase and its hormones. It can be anticipated that differences will be observed in the rate and mechanism for the deactivation of peptides as compared with the catecholamines (32).

Studies with phospholipase-C and liver membrane cyclase suggest a functional role for phospholipids in hormone binding (33). Cyclase activity and glucagon activation are lost following exposure of membranes to phospholipase-C and can be partially restored by replacing phospholipids.

Fat cell cyclase is unique in exhibiting responsiveness to a variety of hormones (e.g., ACTH, vasopressin, glucagon, and epinephrine) (34). When two hormones are used in combination, activation is not strictly additive, suggesting that different hormone receptors regulate the cyclase activity of a single catalytic element (35). Evidence for separate glucagon and epinephrine cyclase systems in liver (31, 36–38) indicates that any given catalytic element responds exclusively to a single hormone. The multi-receptor cyclase systems in fat pose interesting problems in topographic relationships between receptor and catalytic elements. (See description of rhodopsin and photoreceptor adenyl cyclase below.)

In fat cells, increased concentrations of magnesium obliterate hormonal stimulation of cyclase. Thus, hormone stimulation is explained exclusively in terms of the facilitation of magnesium binding to an activator site (39). In the liver, however, hormone stimulation is superimposable over a great range of magnesium concentrations and thus activation by epinephrine or glucagon is not explained in these terms (38). In addition, the binding and activation of adrenal cyclase by ACTH cannot be explained in terms of facilitated magnesium binding (40).

Work with photoreceptor adenyl cyclase and the cyclase systems in brain and testis has suggested that hormone regulation of cyclase is achieved via relaxation of inhibitory constraint. In a number of systems (e.g., brain and testis), homogenate adenyl cyclase is fully active and insensitive to hormone (41, 42) as a result of the mechanical trauma of homogenization. Loss of hormonal response is associated with an enzyme which is fixed in the "on" position. In slice preparations of brain and testis, where cellular architecture is preserved, these tissues exhibit virtually no cyclase activity in the absence of hormone and full responsiveness to added hormones (42–44).

Photoreceptor adenyl cyclase is active in the dark and is inactivated by light (10). The activity of bovine rod disk cyclase is inversely proportional to the fraction of rhodopsin molecules bleached (45). Following exposure of bovine rod cyclase to digitonin, there is a loss of light sensitivity but retention of full cyclase activity and the photochemical properties of rhodopsin (46, 47). Exposure to detergent thus uncouples the regulatory (rhodopsin) and catalytic elements of the photoreceptor cyclase without damaging the intrinsic biochemical properties of either. Again, in the absence of regulation, the uncoupled cyclase system exhibits full enzymic activity, suggesting that in the native system regulation is provided via inhibitory constraint. This hypothesis is also compatible with the evolutionary argument that development of competent enzyme function must occur prior to the acquisition of regulatory control.

Other studies with photoreceptor cyclase have suggested that there may be many rhodopsins (regulatory elements) per (cyclase) catalytic moiety (46). With ratios of rhodopsin to cyclase as large as 100 or 1000 to 1, packing problems would preclude contiguity between regulatory and catalytic elements and therefore argue against classical subunit interaction (48). Rather, the data suggest a loose coupling between regulatory and catalytic elements and the possibility that additional intermediates (e.g., cations) are needed to communicate regulatory influences to the catalytic elements.

PHOSPHODIESTERASE ACTIVITY

The importance of sensitivity in hormone regulation is self-evident; the cyclase system would not provide responsive control if cAMP persisted in the cell well beyond the period of hormone-activated synthesis. A cAMP phosphodiesterase (which hydrolyzes cAMP to 5′AMP) has been found in all tissues containing cyclase in amounts proportional to the levels of cyclase (49). In brain as much as 70 percent of the enzyme is affiliated with the membrane particles (50), while in liver the enzyme is both particulate and soluble (51). Bovine rod disks exhibit a phosphodiesterase primarily located in the disk membrane and which, in contrast to cyclase, does not exhibit light sensitivity (45). The prevailing view depicts phosphodiesterase as a continually active enzyme which provides rapid destruction of formed cAMP. Recent observations, however, have suggested the possibility of regulation at the level of phosphodiesterase. Insulin can increase the activity of phosphodiesterase (52, 53), and this may, in part, explain observations that insulin can lower cAMP levels in liver and fat (54, 55).

Regulation of phosphodiesterase by physiological concentrations of calcium has been related to a phosphodiesterase-activating protein in brain (56). Fluctuations in calcium concentrations in response to changes mediated by cAMP in membrane permeability could thus be an important determinant of cAMP levels in brain and muscle (57). Calcium mimics the effects of light in vertebrate photoreceptors (58). Since adenyl cyclase is present in the photoreceptor disk membrane, calcium effects on disk phosphodiesterase could have an important role in photoreceptor function. Electron micrographs localize phosphodiesterase close to the post-synaptic membrane (59), which supports a post-synaptic role for cAMP in nerve function.

REGULATION OF PROTEIN KINASE BY CYCLIC AMP

A variety of enzymic systems participate in the specific cell responses elicited by cAMP. These responses must occur rapidly since phosphodiesterase prevents prolonged accumulation of cAMP. The effects of cAMP on the activity of a large number of protein kinase systems have been described in great detail. In muscle, cAMP stimulates the activity of a protein kinase which, in turn, activates phosphorylase kinase (60). The latter enzyme then activates phosphorylase by transfer of phosphate residues to serine hydroxyl groups (61). The same protein kinase initially activated by cAMP can also phosphorylate glycogen synthetase, rendering it relatively inactive (the "dependent" form) and thus inhibiting glycogen synthesis while concomitantly promoting glycogen breakdown (62).

Cyclic AMP mediates the lipolytic effects of epinephrine and other hormones in fat cells (2, 63). Recently it has been shown that there is a cAMP-dependent protein kinase which activates lipolytic enzyme systems in fat (64). Initial studies of the role of protein kinase in fat utilized muscle protein kinase. A protein kinase derived from fat, however, functions equally well in the regulation of lipolysis by cAMP (M. Rizack, personal communication).

The molecular events in the activation of protein kinase by cAMP have been beautifully elucidated in adrenal tissue (65). Here the protein kinase consists of two subunits, a cAMP-binding protein (regulatory subunit) and a catalytic subunit. Cyclic AMP binds to and changes the conformation of the regulatory subunit which then dissociates from the catalytic component. The free catalytic moiety can then assume its active conformation. The same mechanism has also been shown for protein kinases in skeletal and cardiac muscle and other tissues (66, 67). In these reactions, cAMP does not act as an energy donor but rather as an allosteric modifier of protein kinase.

Although histones are commonly used as in vitro substrates for protein kinase (68) its physiological substrate is not defined. In brain, phosphorylation of membrane structures by protein kinase has been demonstrated (69). In kidney, vasopressin evokes changes in membrane permeability by this mechanism (70). Microtubular protein has also been suggested as a physiological substrate for protein kinase in brain (71). A relation between protein kinase function and protein synthesis has been demonstrated in adrenal where a specific ribosomal protein is phosphorylated in response to cAMP. This results in a marked increase in the rate of translation of messenger RNA into protein (72).

Suggested physiological receptors for cAMP-mediated phosphorylation reactions thus include phosphorylase kinase in liver, microtubular protein in brain, plasma membrane structures in kidney and brain, and a ribosomal protein in adrenal. The variety of proteins which can be phosphorylated by cAMP-activated protein kinase parallels the large number of effects elicited by adenyl cyclase hormones.

Experiments with cytochalasin have shown that microfilaments are involved in melanosome dispersion (73). Thus, since both melanosome dispersion and melanocyte stimulation are mediated by cAMP (3, 4) these observations suggest interactions between cAMP-dependent enzymes and the microfilament system.

Cyclic AMP can also effect reactions in *E. coli* which are independent of phosphorylation. Cyclic AMP regulates protein synthesis in *E. coli* by binding to a specific protein which governs transcription of the *lac* operon without influencing phosphorylation reactions (74).

Cyclic AMP can affect calcium levels (via membrane permeability changes) and thus calcium has been suggested as a "third messenger" in the cyclase system (75). This sequence of events would involve phosphorylation reactions which produce changes in membrane permeability which precede changes in calcium flux. The effects of calcium on phosphodiesterase (vide supra) permit very effective feedback control of cAMP levels in a variety of cells.

There is also evidence that stimulation of lipolysis (which is mediated by cAMP) can stimulate the release of prostaglandins (76). Prostaglandins have a wide range of effects including stimulation of adenyl cyclase in a number of tissues (vide infra). The prostaglandin cyclase axis may constitute another amplifier device in the cyclase cascade.

The role of phosphorylation reactions in adenyl cyclase function implies the need for a phosphatase to reverse hormone effects and restore a basal or responsive state. Earlier studies in liver showed that a specific phosphatase could reverse phosphorylase activation (77). More recently, this enzyme was shown to be inhibited by cAMP, further extending regulation and amplification into all levels of the cyclase cascade (78).

CALCIUM AND ADENYL CYCLASE

Possible interactions of calcium with the adenyl cyclase system have been the subject of much speculation. An interesting model suggests that cAMP accomplishes regulation by controlling calcium flux (75). In many of the systems in which cAMP has profound effects (nervous system, muscle, and photoreceptors) (58, 79–81) calcium also plays a prominent role in excitation and membrane physiology. Calcium could act as a derivative or third messenger in the sequences which are regulated by cyclase. Striking effects of calcium on phosphodiesterase activity (56) provide additional possibilities for regulation by this cation.

EFFECTS OF CYCLIC AMP ON PROTEIN SYNTHESIS

Many adenyl cyclase hormones (e.g., TSH and ACTH) have profound effects on the size and weight of target tissues. One of the most complete sequences describing the effects of cAMP on protein synthesis emerges from studies with adrenal (vide supra). In response to ACTH, cAMP synthesis is stimulated and mediates the phosphorylation of a ribosomal protein which regulates translation (72). Although this work supports a role for cAMP at the level of translation, effects on transcription are also described. Cyclic AMP is thought to affect protein synthesis by the phosphorylation of histone serine hydroxyl groups in liver (82). Cyclic AMP stimulates protein synthesis in thymic cells and erythroid precursors presumably via histone phosphorylation and effects on transcription (83). There is, however, no direct evidence that histone phosphorylation can cause an increase in protein synthesis or that histones are an important physiological substrate for protein kinase.

In *E. coli* there is a cAMP-binding protein which is directly involved in facilitating transcription of the *lac* operon. This protein acts in the operator region of the *lac* gene and permits transcription only in the presence of cAMP (74). The effects of cAMP on protein synthesis are relatively slow compared to its explosive effects on substrate mobilization, hormone release, and changes in membrane permeability (1, 2, 5–8, 70).

ADENYL CYCLASE AND THE CENTRAL NERVOUS SYSTEM

There are high levels of adenyl cyclase, phosphodiesterase, and protein kinase in nerve tissue, and cAMP plays an important role in metabolic and synaptic transactions in brain (40, 43, 44, 84). Studies using brain slice preparations have shown that neurohumoral agents regulate synthesis of cAMP in nerve tissue (43, 44). Two types of roles have been considered for cAMP in such nerve tissue. In the first, cAMP functions directly as an element in synaptic events, and in the second it functions in a supportive metabolic role (85). These roles are not mutually exclusive and the evidence in support of either is incomplete. A pre-synaptic role for cAMP has been suggested by experiments which indicate that both cAMP and methylxanthines increase the frequency of miniature end plate potentials without producing a change in their amplitude (86). A post-synaptic role for cAMP is suggested by experiments in which norepinephrine, cAMP, or methylxanthines produce slow hyperpolarization of Purkinje cells (87). Cyclic AMP-stimulating hormones also produce a slow hyperpolarization of neurons in the superior cervical ganglion (88, 89). These effects can be prevented by ganglionic blocking agents, suggesting that they are produced at the postsynaptic membrane.

The relative slowness with which cAMP-induced potentials are dissipated has raised doubts about a role for cAMP in rapid synaptic events (85). The duration of cAMP-mediated potential in nerve tissue, however, may simply reflect the rates at which cyclase neurohumoral agents are metabolized, sequestered, or diffuse away from the synaptic cleft (32).

Protein kinase has been emphasized as a component of central importance in nervous tissue (84). The physiological substrate for the protein kinase in brain may be the plasma cell membrane (69) or microtubular protein (71). The phosphorylation of membrane protein elements has been suggested as a mechanism for cAMP control of membrane conductance changes in nerve (70, 90).

PROSTAGLANDINS AND CYCLIC AMP FUNCTION

Prostaglandins are ubiquitous substances, derived from prostanoic acid and ultimately dietary linoleic acid (91). They stimulate the accumulation of cAMP in cardiac muscle, adipose tissue, thyroid, corpus luteum, kidney, pituitary, ovary, thymus, brain, liver, lung, adrenal, platelets, and other tissues (92–100). Although prostaglandins stimulate cAMP formation in the intact fat pad, they paradoxically inhibit its formation in isolated fat cells (101). Prostaglandins also affect sodium transport in human erythrocytes in which cyclase activity has not been demonstrated (102). The physiological role of the prostaglandins is uncertain since it is not clear how their synthesis is regulated. Cyclic AMP-mediated lipolysis could enhance prostaglandin synthesis and thereby provide amplification of the lipolytic stimulus (103). Inhibitory effects of prostaglandins on phosphodiesterase have also been suggested (104).

The synthesis of prostaglandins is blocked by aspirin and this is thought to underlie its anti-inflammatory effects (105). Use of prostaglandin inhibitors as anticoagulants (especially in patients with valve prostheses) appears promising and presumably relates to prostaglandin effects on platelet cyclase (106). Prostaglandins are used as abortifacients and ovulatory stimulants and mimic the effects of luteinizing hormone on ovarian cyclase (107). In spite of their neophyte status, the prostaglandins exhibit much promise for new basic insights and exciting clinical applications.

Hunger and the Teleology of Cyclic AMP

A great many organisms respond to a diminished supply of glucose with an increase in synthesis of cAMP. This is true not only in fish, fowl, and man, but is also true in *E. coli* and the cellular slime molds (108). This remarkable observation links a great number of phyla. In bacteria, in the presence of glucose, by some as yet unknown mechanism, cAMP synthesis is diminshed and transcription of the *lac* operon is blocked. The glucose-starved organism has abundant cAMP and transcribes the *lac* operon in order to utilize alternate substrates (74). In the cellular slime mold, glucose deficiency is followed by an increase in cAMP levels. At first this results in a search for new sources of food. In the absence of a new food supply cAMP eventually causes the free-living amoebae to aggregate into multicellular sporulating forms which permit survival during the period of substrate deprivation (109). In man, glucose deficiency results in the stimulation of hepatic cyclase by glucagon. Newly synthesized cAMP stimulates both gluconeogenesis (110) and glycogenolysis.

This remarkable similarity of function for cAMP in species as diverse as man and the cellular slime molds, and its crucial function in intercellular communication suggest an important role for the cyclic nucleotide in phylogenic and ontogenic sequences. Furthermore, the universality of cAMP as a response to hunger suggests its presence in the earliest of self-replicating forms.

Adenyl Cyclase in Disease Processes

The genetics and clinical characteristics of nephrogenic diabetes insipidus (NDI) were characterized even before cyclase function was understood (111, 112). These patients have normal or elevated serum levels of vasopressin and fail to respond to exogenous hormone. Cyclase is normal in all other tissues, including parathyroid-responsive renal cortical cyclase. Thus, there is a focal lesion either in renal medullary cyclase or its vasopressin receptor. Hereditary NDI has been studied in mice. Parathormone response (renal cortical cyclase) is normal in these animals but medullary cyclase is diminished and response to vasopressin absent (70, and T. Dousa, H. Valtin, personal communication). Nephrogenic diabetes insipidus can be induced in rats with 2-amino-4,5-diphenyl thiazol. These animals have the same focal lesion in renal vasopressin-responsive cyclase as do mice with hereditary NDI (113).

Pseudo-hypoparathyroidism (PHP) is an analogous cyclase disorder in which patients have normal or elevated parathyroid hormone levels, and nevertheless have calcium abnormalities including exostoses, hyperphosphatemia, hypocalcemia, skeletal deformities, and occasionally CNS calcification. They are refractory to exogenous parathormone which fails to produce a cAMP-uria and a phosphaturia (114, 115). In one instance, renal tissue from a PHP patient responded to parathormone in vitro, but the methods of enzyme preparation may have obscured the receptor-cyclase lesion (116).

In some cases of thyrotoxicosis elevated levels of long-acting thryoid-stimulating hormone (LATS) have been described. This is a gamma globulin, synthesized in the liver that selectively activates thyroid adenyl cyclase (117). In these patients, thyrotoxicosis results from the inappropriate stimulation of thyroid cyclase by LATS.

The unusual secretion of sodium and fluid from the gut in cholera enteropathy results from inappropriate stimulation of intestinal cyclase by cholera toxin (118). The effects of cholera toxin on hepatic cyclase mimic epinephrine. Washed liver membrane particles, prepared from rats given cholera toxin intraperitoneally, exhibit an epinephrine cyclase which is fully activated and does not respond to additional epinephrine. The glucagon response is unaffected (119). Cholera-intoxicated hepatic epinephrine cyclase resembles that cyclase in which regulatory and catalytic elements are uncoupled and inhibitory constraint is lost. This suggests that cholera toxin can produce structural changes which damage the coupling between catalytic and regulatory elements. Structural changes are also suggested since the effects of in vivo cholera toxin on liver cyclase survive preparation of washed membrane particles.

The effects in liver also suggest the possibility of a catecholamine-sensitive cyclase in gut which loses regulatory constraint as a result of cholera toxicity. The failure of toxin to affect glucagon cyclase in liver suggests structural differences in the membrane environment of the two liver cyclases.

Studies in mice have shown that increased sensitivity to catecholamines in animals treated with endotoxin is associated with an increase in epinephrine-stimulated cyclase activity (120). In view of the importance of adenyl cyclase for platelet function, glycogen metabolism, fat metabolism, and synaptic function (to name but a few), it must be anticipated that additional disease states will ultimately be explained by cyclase malfunction.

SUMMARY

Many hormones specifically regulate the activity of cyclase which occupies a central role in mammalian cellular communication processes. Cyclase has been implicated in functions as diverse as visual excitation and slime mold aggregation. Cyclase appears in many phyla and may be involved in the developmental transition from single to many-celled organisms. Cyclase hormones appear to function by relaxing inhibitory constraint. Cyclic AMP acts as an allosteric modifier and promotes the phosphorylation

of a variety of proteins, altering membrane permeability and Ca^{++} flux. There is enormous promise for the clinical and pharmacological application of cyclase information and compounds which can selectively regulate cyclase or its derivative enzyme systems.

THE MECHANISM OF ACTION OF INSULIN

LOCUS OF ACTION AND BINDING CHARACTERISTICS

Although the details of insulin action remain elusive, recently some fundamental questions have been answered. For example, does insulin work at the surface of the cell or must it penetrate the cell membrane in order to produce metabolic effects? This question was studied with the ingenious technique of linking insulin covalently to sephrose beads whose size precludes cell entry. Insulin-sephrose produces the entire range of insulin effects in isolated fat cells (121). The effects of insulin could be reversed by treating the cells with anti-insulin antibody, and firm binding of insulin to fat cell receptors was shown with affinity chromatography. Using labeled insulin, the binding characteristics and specificity of insulin sites in liver have been studied in excellent detail (122).

EFFECTS ON CYCLIC AMP LEVELS

It is tempting to postulate that insulin interferes with the synthesis of cAMP, since reduced cAMP levels could explain all insulin effects (stimulation of glycogen synthesis, inhibition of lipolysis, glycogenolysis, and gluconeogenesis). Acute, direct inhibitory effects of insulin on adenyl cyclase have not been demonstrated. In muscle, insulin can stimulate glycogen synthesis under conditions where cAMP is either steady or increasing (123, 124). Insulin-mediated reduction in the levels of cAMP in liver and fat is demonstrable only when cAMP levels are first elevated by glucagon, epinephrine, or other hormones (125, 126). In liver, although insulin does not directly influence cyclase activity, the administration of anti-insulin antibodies in perfused liver produces an increase in cAMP levels.

It has also been suggested that insulin stimulates the synthesis of a second messenger independent of cAMP (e.g., cyclic GMP) which interacts or competes with cAMP (127). This hypothesis provides a mechanism by which insulin can indirectly antagonize the function of cAMP without directly affecting its synthesis.

REGULATION OF EPINEPHRINE-SENSITIVE HEPATIC CYCLASE

In diabetic rats there is a marked increase in the level of hepatic epinephrine-responsive cyclase activity while the activity responsive to glucagon is unchanged (128, 129). In addition, chronic administration of insulin reduces the level of epinephrine cyclase by more than 80 percent. Thus, ambient insulin levels determine the levels of epinephrine cyclase in liver. Effects of insulin on epinephrine cyclase activity do not occur with sufficient speed to account for minute-to-minute regulation of blood glucose. Nevertheless, effects of insulin on epinephrine cyclase constitute an additional means by which insulin regulates glucose metabolism (129).

EFFECTS ON OTHER ENZYMES

It has also been suggested that insulin can reduce cAMP levels either by stimulating phosphodiesterase or by facilitating the release or sequestration of cAMP. Stimulation of phosphodiesterase activity (130) in fat cells by insulin has been reported.

Inhibitory effects of insulin on protein kinase in rat diaphragm have been found (131). These effects appear to be independent of adenyl cyclase activity, since they occur without change in total cAMP content. The molecular mechanism of these effects is not known.

The C-peptide of insulin, which appears as a derivative of enzymic cleavage of proinsulin, has no known metabolic function, and there are no metabolic consequences following the administration of anti-C-peptide antibody (132).

SUMMARY

Insulin functions from the surface of the cell and this necessitates the existence of a second messenger to mediate effects on intracellular enzymes. Candidates for the second messenger include a cyclic nucleotide or some as yet uncharacterized intermediate. Although insulin affects a number of cyclase derivative enzymes, the mechanism of these effects is still obscure. The characterization of insulin effects in precise molecular terms remains a basic problem in understanding the pathogenesis and improving the treatment of diabetes.

MECHANISM OF ACTION OF THYROID HORMONES

OXIDATIVE PHOSPHORYLATION

Thyroxin affects cytoplasmic-ribosomal protein synthesis, transcription, the synthesis of mitochrondrial cytochromes and structural proteins, and mitochondrial respiratory function. The earliest effect of thyroxin follows the appearance of tetraiodothyronine (T4) iodine in isolated mitochondria prepared from hypothyroid animals. Physiological concentrations of T4 produce marked increases in oxidative phosphorylation which occur as early as 2 min following the administration of thyroxin and which are proportional to the log of the concentration of mitochondrial thyroxin (133). These effects of T4 occur in the absence of any significant deiodination in the preparations examined (F. Hoch, personal communication). The rapid accumulation of thyroxin in mitochondria and the associated effects on oxidative phosphorylation are quickly reversed by the addition of bovine serum albumin which removes thyroxin from the treated mitochondria (134).

The effects of thyroxin on oxidative phosphorylation have been explained in terms of a hypothetical high-energy nonphosphorylated intermediate designated "C" (135). The concentration of "C" is rapidly and reversibly affected by the presence of thyroxin in mitochondria. When thyroxin is present in normal concentrations, "C" acts as an energy reservoir and permits loose coupling between respiration and oxidative phosphorylation. When thyroxin is present in toxic amounts it can apparently promote hydrolysis of the high-energy bond between "C" and a carrier molecule, resulting in thermal dissipation of energy rather than its conservation in bio-utilizable "energetic" molecules (e.g., ATP) (136). Thus, in normal concentrations, thyroxin loosens mitochondrial coupling and expedites substrate oxidation and, in toxic amounts, uncouples oxidative phosphorylation and prevents transfer of energy to biologically utilizable intermediates.

In the hypothyroid animal, a deficiency of thyroxin is associated with reduced concentrations of "C," and therefore respiration is tightly coupled to oxidative phosphorylation. In the absence of "C" substrate, oxidation depends absolutely on the availability of ADP and P_i, and though efficient, is markedly reduced.

These acute effects of thyroxin and the associated hypothetical role of the high-energy intermediate "C" explain both the physiological and toxic effects of thyroid hormones on mitochondrial metabolism. A criticism of this model concerns the double role postulated for thyroxin, which governs the availability of "C" and, at higher concentrations, promotes the hydrolysis of "C" from carrier molecules without associated phosphorylation. This is not a damaging objection, however, since thyroxin, in fact, does demonstrate quantitatively different effects depending on the concentrations which prevail, and this is compatible with different thyroxin affinities for each of two binding sites.

Effects of Triiodothyronine

Although T4 has been used exclusively in the experiments showing rapid effects of thyroxin on mitochondrial metabolism, there is convincing evidence that triiodothyronine (T3) contributes a significant amount to the metabolic effects of thyroid hormone in man. Using radioimmunoassay and gas chromatography, serum levels of T3 have been measured, and these measurements in combination with estimated turnover rates have been used to calculate the total quantity of T3 produced by the human thyroid gland (137). Based on these calculations it is clear that T3 makes a significant contribution to the metabolic effect of thyroid hormones.

Effects on Protein Synthesis

Thyroxin produces a profound and rapid stimulation of ribosomal cytoplasmic protein synthesis which is mediated by a nonprotein, non-nucleic acid, heat-stable, water soluble substance which thyroxin releases from mitochondria within 5 min following its administration in vitro (138). Thyroxin also produces a marked increase in ribosomal protein synthesis in vivo within 2 hr following administration (139). The effects of this mitochondrial factor on ribosomal protein synthesis have been studied under conditions which exclude artifacts of pH and ionic strength.

Ribosomes from the liver of hypothyroid animals show deficient protein synthesis in vitro (140). In addition, mitochondrial protein synthesis is depressed in the hypothyroid animal (141, 142). Physiological doses of L-T3 can produce stimulation of protein synthesis by cytoplasmic ribosomes which peaks 48 hr following administration of the hormone to intact animals (143, 144). In these experiments, T3 produces a general stimulation of protein synthesis and a specific increase in the synthesis of mitochondrial proteins including cytochrome *c* which is deficient in hypothyroid animals. The fall in basal metabolic rate in the hypothyroid animal precedes the fall in cytochrome *c* content in mitochondria (145). Thyroid hormones also stimulate the uptake of amino acids by mitochondria in vitro.

Thyroid hormone administered in vivo affects the synthesis of nuclear messenger RNA. Within 3 to 16 hr following administration, there is a rise in the metabolism of the nuclear RNA fraction (146, 147).

EFFECTS ON RIBOFLAVIN METABOLISM

Thyroid hormones influence the metabolism of riboflavin, especially the conversion of riboflavin to flavin adenine dinucleotide (FAD) (148). In hypothyroid animals, this conversion and the levels of FAD and flavin mononucleotide (FMN)-containing enzymes are markedly diminished. The hypothyroid state is also associated with reduced levels of those enzymes which metabolize FMN and FAD. The induction of mitochondrial α-glycerophosphate dehydrogenase by thyroid hormones is limited by the availability of FAD and cannot be demonstrated in riboflavin-deficient animals (149). Similarities noted between hypothyroidism and riboflavin deficiency derive from the fact that the enzmes which regulate the synthesis of FMN and FAD are under the control of the thyroid hormones (150).

SENSITIVITY TO CATECHOLAMINES

Increased sensitivity to L-epinephrine in the thyrotoxic state has long been noted. Increases in the levels of adenyl cyclase in the fat pad by thyroid hormone could explain this observation (151).

SUMMARY

The immediate action of the thyroid hormone is a rapidly reversible effect on the level of a nonphosphorylated, high-energy intermediate, which permits loose coupling or respiration and oxidative phosphorylation. A derivative effect of thyroid hormone is the release of a mitochondrial substance which stimulates the synthesis of protein by cytoplasmic ribosomes, and is involved in subsequent effects on messenger RNA synthesis, mitochondrial protein synthesis, and riboflavin metabolism. More work is needed to delineate accurately the mechanism of effects on protein synthesis, the initial point of contact between the thyroid hormones and mitochondria, and the nature of participating high-energy intermediates. In spite of the problems remaining, an excellent beginning has been made in the task of integrating these various effects into a coherent sequence.

STEROID HORMONES

INTRODUCTION

Steroid hormone effects in a variety of systems result from stimulation of specific and general protein synthesis. Effects of steroid hormones are found at the level of both transcription and translation. The cortisone-mediated induction of alkaline phosphatase in fibroblasts is an exception to this generalization, and reflects a change in the kinetic characteristics of the enzyme rather than an increase in the number of molecules synthesized (152).

TRANSCRIPTIONAL EFFECTS

Estrogen steroids increase the activity of nuclear RNA polymerase and nuclear messenger RNA in the chick oviduct system (153, 154). Androgens are equally effective in stimulating nuclear messenger RNA synthesis in prostate and seminal vesicles (155). The insect steroid ecdysone produces readily visible "puffing" in the giant chromosomes of the fruit fly salivary gland. Ecdysone-induced puffing is followed by a burst of nuclear RNA synthesis (156).

The aldosterone-mediated stimulation of electrogenic sodium pumping in toad bladder and kidney is prevented by compounds which block the synthesis of messenger RNA or protein, and exhibits a time course which parallels the stimulatory effects of aldosterone on protein synthesis (157, 158). The technique of DNA hybridization permits the conclusion that the increased amounts of nuclear messenger RNA synthesized by chick oviduct in response to estrogenic steroids are composed predominantly of new species of RNA (159). These findings rebut the criticism that the stimulation of messenger RNA synthesis by steroid hormones is a general rather than a specific effect and indicate that estrogens cause expression of a unique portion of the genetic information.

STEROID-BINDING PROTEINS

Tissues that are sensitive to steroids contain cytoplasmic steroid-binding proteins which exhibit hormonal specificities compatible with the steroid sensitivity of the tissue. The cytoplasmic steroid-binding proteins serve a vehicular function and facilitate entry of steroid molecules into the nucleus where they are bound by nuclear steroid-binding proteins. Profound similarities exist between the specificity of binding for cytoplasmic and nuclear proteins that bind cortisone in liver cells adapted to tissue culture (160). Similar binding proteins are involved in the function of a variety of other steroid hormones (161–165).

Using tritiated aldosterone as a marker, specific binding proteins have been found in the cytoplasm of kidney cells (166) which exhibit convincing specificity for aldosterone and related mineralocorticoids. Translocation of tritiated aldosterone from cytoplasm to nucleus depends on the presence of the cytoplasmic binding proteins which serve a vehicular function. In the absence of this cytoplasmic binding protein, penetration of labeled aldosterone into isolated nuclei is markedly diminished (167). Following translocation of tritiated aldosterone into the nucleus there is binding by nuclear chromatin material, associated with stimulation of messenger RNA synthesis (168).

The penetration of steroids into the nucleus is followed by affiliation with nuclear binding proteins, stimulation of nuclear RNA polymerase, and increased RNA synthesis.

EFFECTS ON TRANSLATION

Steroid hormones can also affect protein synthesis at the level of translation (169, 170). Evidence includes experiments in isolated hepatoma cells in tissue culture showing marked effects on enzyme induction in the absence of increased messenger RNA synthesis. Induction of tyrosineaminotransferase (TAT) by cortisone can occur in tissue culture hepatoma cells in the absence of increases in general protein synthesis (171). In systems where TAT synthesis is increased as much as 15-fold, there is no change in total RNA synthesis, the turnover of rapidly labeled RNA, or the activity of RNA polymerase (172, 173).

Using actinomycin D in concentrations which completely block RNA synthesis, one can show a superinduction of TAT under conditions in which there is no effect on its rate of degradation (174). Under the conditions for superinduction of TAT in hepatoma tissue culture with actinomycin D, the synthesis of TAT becomes constitutive rather than inducible and the steroid inducer may be removed without a reduction in enzyme synthesis. In experiments in which messenger RNA synthesis is entirely inhibited by actinomycin D and the inducing steroid is removed, there is no immediate decline in the rate of synthesis of TAT, indicating relative stability of its messenger RNA (175).

These observations argue against the classical Jacob-Monod model for enzyme induction as a phenomenon which is explained entirely in terms of transcriptional control (176). A different model has been proposed which depends upon a hypothetical post-transcriptional repressor, exhibiting the biochemical characteristics of a rapidly turning over, labile protein (169, 170). This protein is assigned two functions: first, it serves as a translational repressor by binding to a specific mRNA and blocking its translation into enzyme protein. In addition, labile repressor markedly increases the propensity for the destruction of its specific mRNA. This could simply result from the relative protection afforded mRNA during translation and its increased vulnerability to RNAase when translation is blocked by labile repressor. When mRNA synthesis is completely blocked, the messenger for the synthesis of labile repressor and the labile repressor itself rapidly decline. This would permit stabilization and continuing accelerated translation of existing TAT mRNA. Insulin can also stimulate TAT synthesis by a mechanism which is independent of transcription, and these effects are additive to those of cortisone (173, 176, 177).

REMARKS

These and related observations support the idea that the fine control of protein synthesis by steroids occurs in part as a result of effects on translation. Much work is needed to reconcile the evidence for steroid effects on transcription and translation and to identify hypothetical elements in translational control (e.g., labile repressor). It is emphasized that transcriptional and translational steroid effects on protein synthesis are not mutually exclusive but together afford great flexibility. Transcriptional effects could predominate in developmental stages while both transcriptional and translational effects occur in the fully differentiated cell (177).

SUMMARY AND CONCLUSIONS

The molecular intermediates which participate in hormone action have been reviewed. The molecular-enzymic events which underlie clinically observed hormone effects are apparent in some cases. The effects of glucagon on hepatic cyclase resulting in accelerated glycogenolysis (178) and gluconeogenesis (110) account, in a reasonable way, for the associated hyperglycemia. The effects of thyroxin on the coupling of respiration and oxidative phosphorylation explain the calorigenic and toxic effects of differing amounts of thyroid hormone (136). However, relationships between the known effects of thyroxin on mitochondrial metabolism and protein synthesis are less satisfying as explanations for the changes in skin and its appendages associated with fluctuations in hormone levels. The effects of thyroxin on the levels of epinephrine-responsive cyclase could explain the increased sensitivity to catecholamines in the thyrotoxic state (151).

The connection between effects of ACTH, epinephrine, glucagon, and insulin on cAMP levels in fat and derivative lipolytic or antilipolytic effects seems clear (126). The relationship between cAMP and calcium sequestration by the sarcoplasmic reticulum could explain the inotropic and chronotropic effects of catecholamines (179, 180). The effects of cAMP on microtubular function (71) suggest a mechanism by which melanocyte-stimulating hormones can influence the migration of melanosomes (3, 4).

For many hormones, including insulin, the steroids and thyroxin, there is not yet a sufficient description of the elements in the responding set. Stimulation of adenyl cyclase by hypothalamic releasing substances (5) and beta cell cyclase by glucagon (6) in the appropriate setting, lead inevitably to the release of pituitary hormones and insulin, respectively.

Although a role for Ca^{++} seems likely, the nature of the machinery which translates cyclic AMP levels into the release of hormone packets remains elusive. Time and much work are required before the details of these events can be suitably reconstructed to permit a complete sequential description, from the moment of initial interaction between the hormone and its receptor to the final appearance of grossly observable clinical and physiological effects. Nevertheless, it is realistic to expect rapid strides in the immediate future. The milieu of supporting information and methodology favors the likelihood of enormous growth in the level of understanding, and the potential for correction of human endocrinopathies.

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ABETALIPOPROTEINEMIA

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Introduction

This rare, inherited disease has attracted wide interest since the first patient was described a little more than 20 years ago (1). In the past two decades, a total of 25 patients have been considered to share the same genetic defect. The manifestations of this disease include abnormalities in the lipid and protein composition of the plasma lipoproteins, abnormal morphology and lipid composition of the red blood cells, morphologic changes in the intestinal villi with impairment in fat absorption, retinitis pigmentosa, and involvement of the central nervous system. Many investigators have been interested in studying one or more aspects of this disease, and several review articles have appeared which adequately review case reports and clinical investigations (2–6). In this article, in addition to a general review, I wish to concentrate upon the studies which are concerned with the elucidation of the abnormalities in plasma lipoproteins, red cells, and gastrointestinal fat absorption. Despite intense effort, there is no satisfactory explanation for the abnormalities in these three systems, and further studies are needed to answer the problems presented.

The most commonly accepted label for this disease is at present abetalipoproteinemia (ABL). The initial case reports stressed the abnormalities in the shape of the red blood cells, and the early literature listed these patients under the term acanthocytes or acanthocytosis, or by the name of the authors of the first case, Bassen-Kornzweig syndrome (7–9). Approximately 10 years after the first case report, several groups noted that the low density or beta lipoproteins were absent from the plasma of their patients (10–13). The terminology was therefore further modified on the supposition that this lipoprotein abnormality might be the basic defect, and the term congenital β-lipoprotein deficiency was employed (3). However, the deficiency of beta lipoproteins, when studied by electrophoresis and immunologic testing, appeared to be complete, and in an effort to distinguish these patients with complete absence of betalipoprotein from a group of patients with hypobetalipoproteinemia, the term abetalipoproteinemia was chosen (2). More recent studies suggest that we may be able to further redefine the plasma lipoprotein abnormalities; but for the present, ABL seems to be the most useful label for these patients.

CLINICAL FEATURES

Although there is considerable variation in the extent of neuromuscular involvement in the case reports, the description of infancy and childhood in these patients appears to conform to the following pattern. At birth, the infant is apparently entirely normal and the neonatal period is rarely complicated. The infant appears to gain weight and grow more slowly than normal infants, and steatorrhea accompanied by abdominal distension are prominent symptoms. During this period, the diagnosis of celiac disease is made, and dietary modifications are introduced. As the child grows, the requirement for a rigidly fat-restricted diet becomes apparent, and attention to supplementation with the fat-soluble vitamins may be made. Toward the end of the first decade of life, clumsiness and ataxia become apparent, with some diminution in muscular strength. Patients during this phase are frequently misdiagnosed as cases of Friedreich's ataxia. Ocular symptoms of nystagmus and strabismus due to muscle weakness are common findings during this period, and as puberty approaches, visual complaints of scotomata and evident decrease in visual acuity develops due to retinal degeneration with pigment deposit. Early adult life has a more varied clinical syndrome, and several patients have developed cardiovascular abnormalities including disorders of heart rhythm and congestive heart failure. At least six patients have died, and a report of one complete postmortem study has been published (14).

Analysis of the genetic data indicates that the disorder is transmitted as an autosomal recessive and that the affected patients are probably homozygous for a single genetic defect. No abnormalities or partial defects have been found in the heterozygous parents, although one report noted a reduction in the concentration of betalipoproteins in both parents and one grandparent of an affected child (11). There is a relatively high degree of parental consanguinity in affected families; a large proportion of families have been of Jewish and Italian parentage, but other groups have included Negro, French, Scottish, and English lineage. The detection of the heterozygous state depends upon the identification of the basic genetic defect and subsequently the development of a method to detect this abnormality.

PLASMA LIPOPROTEINS

The recognition of abnormally low concentrations of lipids (cholesterol and phospholipid) in the plasma of affected patients led to a more intensive analysis of the plasma lipoproteins. The examination of the plasma or serum in these patients reveals unusual clarity, with absence of the normal slight turbidity or opalescence that characterizes normal plasma. In recent years, the plasma lipoproteins have been intensively studied, and several classifications have been suggested. The two main techniques used to separate (and classify) lipoproteins have been the ultracentrifuge (both preparative and analytical) in which the separation is by the density of the particles, and the electrophoretic system in which separation is determined by the charge on the particles and their movement in the supporting field (15). While there is a fair correspondence in the separation by these two systems of high- and low-density lipoproteins and alpha and beta lipoprotein, respectively, these categorizations are far too broad to reflect the intricate structure and heterogeneity of lipoproteins. Immunologic reactions are most frequently used to detect differences in composition of antigens rather than to establish identity in structure. I have already indicated that the term abetalipoproteinemia, operationally useful at the present time, is probably not the most accurate label for the patients. It is true that electrophoresis of the plasma lipoproteins in these patients will show an absence of beta lipoproteins, and that the antibody prepared to normal human beta lipoproteins will be nonreactive with the plasma of these patients. By ordinary preparative ultracentrifugation, the supernatant of tubes containing plasma spun at density 1.063 for 20 hours at 100,000 X *g*, will be free of lipoproteins classified as very low-density lipoproteins (VLDL), and low-density lipoproteins (LDL), and no immunologic reaction will develop with antibodies prepared to normal VLDL and normal LDL with this supernatant. And there is uniform agreement that chylomicrons are never present (in any appreciable amount) in the plasma of these patients whether fasting or after a fatty meal. But recognition of the heterogeneity of the plasma lipoproteins and of the overlap in the polypeptide composition of the lipoproteins has stimulated further analysis of the lipoproteins in these patients.

The protein constituents, or apoproteins, have been studied by delipidation of the lipoproteins obtained by preparative ultracentrifugation (17, 18). Several laboratories have identified polypeptide chains in the apoproteins of the lipoproteins, but, unfortunately, their terminology in referring to these fractions is not uniform. In fact, the different terminology is due to different techniques of separation and even relatively minor methodologic variations will change the apparent distribution of the peptide chains obtained by column chromatography. Further, the present technique for identifying the structure and composition of the peptide chain is at an early stage in its development, and as additional skill is achieved, revision of the present suggested structure will occur. The fractionation of the delipidated lipoproteins by gel filtration (19, 20) ion exchange chromatography (18, 21, 22) or by isoelectric focusing (23) yields different fractions, which have been designated by some groups by their carboxyl terminal amino acids, by others as fractions identified by Roman numerals identifying the operational position from the separating column, and by other groups by detailed amino acid composition analysis after digestion of a fraction. A detailed analysis and comparison of these studies appears premature, but reference will be made to two laboratories that have published extensively on lipoprotein structure, and have studied patients with ABL (17–19, 22–25).

Each lipoprotein class, isolated by preparative ultracentrifugation, appears to have several polypeptide chains, and these can be designated as major and minor components based on their weight content in each lipoprotein class. The laboratories from the National Institutes of Health have designated the apolipoproteins by the carboxyl terminal amino acid as follows: ApoLP-ser is apolipoprotein serine; ApoLP-gln is apolipoprotein glutamine; ApoLP-val is apolipoprotein valine; ApoLP-thr is apolipoprotein threonine; ApoLP-glu is apolipoprotein glutamic (15, 18, 22, 25). In their studies, high-density lipoproteins (HDL) have ApoLP-thr and ApoLP-gln as major constituents, and ApoLP-ala, ApoLP-glu, and probably ApoLP-val as minor constituents. The low-density lipoproteins (LDL) have ApoLP-ser as the major constituent and ApoLP-ala as a minor constituent. The very low-density lipoproteins (VLDL) have ApoLP-ser, ApoLP-val, ApoLP-glu, and ApoLP-ala as major constituents, and ApoLP-thr and ApoLP-gln as minor components. Chylomicrons have been shown to have at least ApoLP-ser, ApoLP-thr, and ApoLP-gln as major constituents. Analysis of the lipoproteins of one patient with abetalipoproteinemia revealed the presence of the apolipoproteins ApoLP-thr and ApoLP-gln (major constituents of HDL), and also ApoLP-val, ApoLP-glu, and ApoLP-ala as minor components, and in roughly the normal amount that might be expected from HDL (25). Gotto et al (25) stressed the absence of ApoLP-ser and suggested that this peptide may be the fraction uniquely required for the release of triglyceride from the intestine and lymph into the plasma, and for the formation and release of VLDL and LDL into the circulation.

Reports from another laboratory, directed by A. M. Scanu, have indicated that the above classification represents an oversimplification of the polypeptide structure of the apolipoproteins, and that additional heterogeneity is present (26). For example, the ApoLP-ala can be found in normal VLDL to exist in three different forms with differing amounts of sialic acid in relation to the protein content. There is a polypeptide which contains two moles of sialic acid per mole of protein; a polypeptide which has only one mole of sialic acid per mole of protein and a fraction that has no sialic acid (18, 19, 22, 23). Furthermore, ApoLP-thr, isolated from normal HDL, does not contain carboxyl terminal threonine, but the terminal amino acid is glutamine (27). Studies by this laboratory were carried out on large amounts of plasma from three patients with ABL. These plasma samples were obtained by plasmaphoresis and separated by preparative ultracentrifugation in the usual manner. Results (as yet unpublished, Lim, Kayden and Scanu) may be summarized as follows: (*a*) no chylomicrons were found; (*b*) small amounts of lipoproteins floating at $d < 1.006$ (VLDL) and d 1.006-1.063 g/ml (LDL) were isolated having as a protein moiety polypeptides with the same mobility and immunologic properties as those of Apo

HDL fraction V (19); (*c*) a significant decrease of the HDL_3 subclass; and (*d*) abnormality in composition of Apo HDL fraction V with a decrease in the sialic acid content affecting one of the polymorphic forms of the peptides having alanine as carboxyl terminal amino acid. These findings support the observation that so-called ApoLP-ser or fraction A+B of VLDL were not found in the isolated lipoproteins. The observation of abnormalities in HDL was not unexpected since Jones & Ways (28) had, in 1967, reported that HDL_3 was present in normal amounts. In this report, these authors also stressed that the phospholipid distribution of both HDL fractions is abnormal, with low concentrations of lecithin and an increased percentage (though normal absolute quantity of sphingomyelin). The abnormalities in phospholipid distribution and amount will be discussed in greater detail in connection with the red cell lipid composition. To conclude this section on the plasma lipoproteins: it appears that a major, if not the major, polypeptide fraction of LDL is absent in the plasma of these patients. This polypeptide fraction is also a constituent of VLDL and of plasma chylomicrons. The precise interpretation of the absence of this polypeptide fraction is uncertain —is ApoLP-ser normally synthesized within cells, but the pathway for synthesis of the lipoprotein blocked by some mechanism? Is ApoLP-ser truly essential for the binding or assembly of lipid to form a lipoprotein? What is the significance of the isolation of minute amounts of VLDL and LDL fractions which lack this major polypeptide fraction? What determines the abnormalities in HDL composition and amount? It has been suggested that the absence of ApoLP-ser can account, in molecular terms, for fat malabsorption and severe hypolipidemia (25), but it is likely that lipoprotein structure and synthesis is far more complex, and that the mechanisms underlying the reported structure and metabolic abnormalities in ABL remain to be clarified.

Recently, the plasma lipoproteins have been studied by electron microscopy (29, 30). The plasma from two patients with ABL were separated by preparative ultracentrifugation into HDL and LDL fractions and studied by electron microscopy. The particles observed in the HDL fraction appeared normal in electron microscopy, with the majority between 65 and 100 Å. The structural appearance of particles in the d. 1.006–1.063 g/ml fraction in ABL were abnormal both in their unique shape and in the packing arrangement. The aggregation of the particles tended to be in stacks with distinct periodicity and length, and occasionally the particles were packed in a cube-like arrangement (31). Studies in these areas by the technique of electron microscopy seem promising.

RED BLOOD CELL ABNORMALITIES

The abnormal shape of the majority of the red blood cells in wet or dried blood smear preparations has been and is a striking observation. It prompted the first case report and warranted the coining of a special word, acanthocytes, derived from the Greek word for spine or thorn, akantha. The abnormal shape is best appreciated in wet preparations (preferably fixed in Dacie's solution of 1 percent formalin and 3 percent trisodium citrate) where the problem of crenation of the red cells during the process of smearing and drying on a glass slide is obviated. This morphology is even better visualized in the scanning electron microscope (32). The acanthocytes respond to a variety of changes in the environment just as do normal biconcave disc-shaped red blood cells; they become crenated when exposed to isotonic saline but resume their previous shape when returned to plasma. They respond to hypo- and hypertonic buffered solutions with reversible changes in shape. Despite intensive study, the explanation for their unusual appearance and shape is not available (32). Furthermore, the specificity of the change in the shape of the red blood cells in ABL and the uniqueness of this abnormality has been questioned. Many observers have sought to lump the abnormally shaped red cells seen in some patients with liver disease ("spurred cells") with the red cells of ABL and to use the term acanthocytes for all red blood cells which have spines or spicules. This would eliminate a useful categorization of morphologic shape, plus biochemical abnormality, since one of the unique features of the red blood cells in ABL is in the lipid composition of the red cell membrane.

The cholesterol content of the acanthocytes is normal or slightly increased and the phospholipid content is also normal or slightly decreased (33). There is a decrease in the lecithin content and an increase in the sphingomelin content of the red cell membrane. These changes reflect the plasma distribution of the phospholipids. This distribution is unique to the red blood cells of patients with ABL and there appears to be merit in confining the term acanthocytes to the red blood cells of these patients.

It should be emphasized that the morphology of the acanthocytes may be indistinguishable from the red cells seen in some patients with liver disease ("spurred cells") (34), the red cells seen in some jaundiced newborn (infantile pyknocytosis), the red cells in metastatic liver disease with hemolytic anemia (spiculated erythrocyte), and probably other red cells in patients with pyruvate kinase deficiency, thrombotic thrombocytopenic purpura, and microangiopathic hemolytic anemia (32).

Acanthocytes have a normal or slightly decreased life span, a normal osmotic and acid fragility and an increased susceptibility to mechanical trauma. Anemia, if present, is mild, reticulocytes are usually minimally elevated and haptoglobin levels are normal or slightly reduced. The acanthocytes appear to carry out all metabolic activities in a manner indistinguishable from normal red blood cells. The abnormal form of the erythrocytes appears to develop after circulation in the plasma. Normoblasts and erythrocytes in the bone marrow have a normal shape, and the cells appear to acquire the abnormal morphology after leaving the bone marrow. The acanthocytes retain their abnormal shape when incubated in normal serum, but neither plasma or serum from a patient with ABL will alter the shapes of normal red blood cells when incubated in vitro. However, transfused normal cells appear to undergo changes in shape during the circulation in patients with ABL. The cells after an exchange transfusion in which 80 percent of the red cell mass was derived from normal donors showed greater than 50 percent acanthocytes (10).

Although the acanthocytes have a normal life span and normal osmotic fragility, studies with cells from untreated patients show increased autohemolysis and increased sensitivity to peroxide hemolysis (35–37). The measurement of serum tocopherol levels in these patients gave virtually zero readings; the sensitivity of the method is approximately 1 percent of normal value. The addition of tocopherol to in vitro incubation mixtures corrected the autohemolysis (36). Supplementation of the diet of patients with ABL with a water-miscible preparation of Vitamin E resulted in protection of the acanthocytes from peroxide hemolysis (37). The changes in fatty acid distribution and phospholipid composition of the erythocytes induced by peroxide in the untreated state were also prevented after vitamin E treatment. Continued administration of tocopherol in high doses has given varying plasma levels in patients with ABL (38). In some, detectable levels have been found in the plasma up to a maximum of 20 percent of normal values; in these patients, tocopherol was found in the red cells from 50 to 100 percent of normal values. In other patients, despite oral supplementation for many months, no detectable levels could be found in either the plasma or red cells. The cells of these patients were able to take up tocopherol to a level equal to normal cells as demonstrated in simple incubation studies. (Kayden and Chow, unpublished results) The explanation for the varied results in different patients is not apparent—the possible cause for the differences include dosage, amount of absorption from the gastrointestinal tract, and the capacity of HDL and nonlipoprotein plasma proteins to transport tocopherol. It appears certain, however, that tocopherol is not a determinant in the morphology of the acanthocyte.

GASTROINTESTINAL MANIFESTATIONS

The presence of steatorrhea and abdominal distension in infancy in patients with ABL has resulted in the erroneous diagnosis of celiac disease. However, dietary change to gluten-free diets does not result in correction of the abnormal bowel movements. The intolerance to fatty food which is a predominant gastrointestinal complaint in infancy and childhood, diminishes during the second decade, and patients learn to adjust their dietary fat consumption. On a diet that is only slightly to moderately restricted in fat intake, steatorrhea disappears, and the fat content of the stool is normal (16). But the pathway for fat absorption is not the normal route of chylomicron formation with passage of the resynthesized triglycerides into the lymph stream. The failure to form chylomicrons is documented by the absence of these particles in the circulation at any time after a fatty meal, and by electron microscopy of jejunal biopsies after such meals (16). The histologic appearance of the intestinal villi is striking; the mucosal cells are filled with lipid droplets, especially at the villous tips and the nuclei of the cells appear pressed to the base of the cells. Fat droplets are virtually absent in the intercellular space or lacteals (3). Biochemical analysis of the intestinal biopsies confirm the presence of increased quantities of triglycerides in the villi (16). Studies with radioactive fatty acids document that the uptake of fatty acids from the intestinal lumen into the intestinal mucosa cells and the esterification of the fatty acids to triglycerides proceed briskly (3). The subsequent fate of the mucosal triglycerides and the ultimate mode of absorption of fatty acids and other lipids remains a matter of conjecture.

Dietary fat in man undergoes emulsification and partial hydrolysis to monoglycerides, and the 2—monoglyceride pathway is the major route of fat absorption during normal digestion and absorption (39). Studies in patients with ABL demonstrate normal pancreatic and biliary function, and normal intestinal enzymatic activity. Evidence from animal models supports the hypothesis that the route of absorption of mucosal triglyceride, when chylomicron formation is inhibited, requires intracellular hydrolysis of the triglyceride with absorption of the released fatty acids via the portal venous stream, presumably as albumin-bound free fatty acids. Sabesin & Isselbacher (40) demonstrated that puromycin limited fat absorption in the rat and produced an abnormal histologic appearance of the small intestine with lipid distension of the mucosal cells of the villi. In a more extensive study of absorption and metabolism of fat in puromycin-treated rats, Kayden & Medick showed that the drug had no effect on the absorption of short-chain fatty acids and triglycerides (41). Long-chain fatty acid absorption into the thoracic duct lymph in the form of chylomicron triglycerides was inhibited by puromycin, but oxidation of the orally administered fatty acids was as complete as in untreated controls (41). The portal venous route for absorption of long-chain fatty acids has been postulated or shown in other animal studies (42–44). That a quantitative deficiency in fat absorption and fat transport in the serum of patients with ABL may exist is demonstrated by studies on the metabolism of the essential fatty acid, linoleic acid. The low values of this fatty acid in plasma phospholipid and cholesterol esters, in red cell phospholipids, in liver triglycerides, and in adipose tissue triglycerides attest to an inadequate quantitative intake. But continued oral supplementation with high linoleic acid triglycerides over many years has resulted in increased adipose tissue triglyceride content of linoleic acid, although not to levels found in normal controls. Whether dietary fatty acids are preferentially oxidized limiting the exchange with the fatty acids synthesized in the body from two-carbon precursors in these patients cannot be answered. It is possible that untreated patients, that is, those without supplementary linoleic acid triglycerides, may reflect some features of essential fatty acid deficiency. It is clear, however, that patients who have been on dietary supplementation do not have any of the biochemical or clinical symptomatology that characterizes essential fatty acid deficiency in man.

If there is concern about the quantitative absorption of essential fatty acids, the problem of fat-soluble vitamins is even more difficult to evaluate. Relatively few problems in blood coagulation have been reported that suggest that there is ever a problem relating to Vitamin K deficiency, although one infant did have severe bleeding requiring hospitalization and K supplementation (45). Considerable attention has been given to the problem of Vitamin A, particularly because of the development of retinal degeneration. Most measurements of plasma Vitamin A levels have shown low value, but on oral supplementation, the values can be raised to normal range. It is not entirely clear what "Vitamin A levels" mean, since these determinations are colorimetric reactions for retinol. In recent years, it has become apparent that Vitamin A is absorbed from the gastrointestinal tract as Vitamin A esters (mostly palmitate) and circulates in the plasma initially in chylomicrons and then mostly in low-density lipoproteins before being stored in the liver as Vitamin A palmitate. However, when Vitamin A is released from the liver, it is as the free alcohol, retinol, and is bound to retinol-binding protein (RPB) , a nonlipoprotein protein that appears to be complexed to albumin and is found in the 1.21 infranatant fraction (46, 47). Preliminary studies on RBP in two patients with ABL have suggested low levels of saturation, but these studies require repetition and amplification (F. Smith and H. J. Kayden, unpublished studies). In a study over many weeks with control of the dietary intake of Vitamin A, a comparison was made of the plasma level of A and retinal response to dark adaptation and the electroretinograms. It was found that after a period of A restriction with a fall in plasma level of A, an abnormal dark adaption response developed. This responded to almost a single oral dose of Vitamin A in one patient, and a relatively rapid response in the second patient. (Carr, R., personal communications). Several patients who are now approaching adolescence continue to have normal retinal function. These patients have been receiving supplementary fat-soluble vitamin medication and also supplementary linoleic acid triglycerides. It is too early to state with security that some of the visual difficulties may have been due to a deficiency state secondary to the intestinal malabsorption. The mode of Vitamin A absorption in the gastrointestinal tract is most challenging. It appears impossible to account for it by the normal chylomicron pathway; can it be absorbed via the portal venous route? Bound to albumin?

Vitamin E metabolism has already been mentioned in connection with red cell lipid composition and sensitivity to peroxide hemolysis (36, 37). The problem of the route of absorption for this vitamin is equally puzzling. Most authors indicate that tocopherol is normally absorbed via the chylomicron thoracic duct lymph pathway, but it is apparent that absorption must occur via some other pathway in these patients. Similarly, the transport mechanism in the plasma requires re-evaluation. In some reports, LDL is considered the major transport system for tocopherol in man, averaging more than 90 percent of total plasma tocopherol. In another report and in recent studies, HDL and even the nonlipoprotein protein appear to transport tocopherol. (Bjornson and Kayden, unpublished studies). The precise role of tocopherol in man remains an unsettled matter. The observation of its role in peroxide hemolysis in patients with ABL have supported other suggestions of the importance of tocopherol in the hematopioetic system. Other areas are more speculative; the role of tocopherol as an antioxidant in man is difficult to establish. A promising area is the possible relation of tocopherol to ceroid pigment deposition in smooth muscle. Ceroid has been considered to represent polymerized oxidized fat, and its presence may signify tocopherol deficiency. In one patient who died at the age of nine, postmortem examination revealed ceroid in the intestinal tract and in the heart. Whether any of the neurological symptoms are related to a deficiency of tocopherol is entirely speculative. In two patients, followed for seven years on high doses of oral tocopherol, there has been no progression of the neurologic symptoms, and their neuromuscular development from the end of first decade to adolescence approaches the normal pattern. It is difficult to state that tocopherol administration has been responsible for this normal development; further support for this suggestion will depend on the observation of other patients who are receiving similar supplements.

The therapeutic aspects of ABL have been concerned with gastrointestinal absorption and other aspects of the syndrome. Exchange transfusion was carried out in one six-year-old child without evident change in the capacity to absorb fat (10). A more extensive study in an eleven-year old boy was carried out which involved the infusion of more than two liters of plasma from a patient with hyperbetalipoproteinemia over a four-day period (48). There was no change in plasma glyceride level, nor any evidence that gastrointestinal absorption was altered. During the period when steatorrhea is prominent, fat restriction has been used with symptomatic relief. In some patients, medium-chain triglycerides have been substituted for ordinary dietary fat, but although their caloric value is greater than carbohydrates, they are poor vehicles for fat-soluble vitamins. In most patients, the capacity to absorb fat increases during childhood and adolescence; it is therefore logical to encourage patients to eat normal dietary fat to the amount that they can tolerate. A diet high in linoleic acid triglycerides and supplemented with Vitamin A and E has been well tolerated by most patients. It will require a very long period of observation to establish whether this dietary modification will contribute toward amelioration or prevention of the abnormalities in retinal function or in the neurologic abnormalities. It is tempting to hope that the neuropathologic changes reported in the one postmortem examination can be prevented by supplementation of dietary components that may be deficient due to gastrointestinal malabsorption.

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ACUTE GASTRIC MUCOSAL BLEEDING[1]

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In 1898 Dieulafoy (1) described a condition characterized by bleeding from superficial gastric erosions. He applied to this condition the term of "exulceratio simplex" to distinguish it from chronic peptic ulcer, a well-known entity at the time. Since then, it has been variously referred to as "stress ulcer," "Curling's ulcer," "Cushing's ulcer," "acute erosive gastritis," and "hemorrhagic gastritis." Until recently, it has often been confused with "peptic ulceration." Since the pathogenesis of these acute, superficial gastric mucosal erosions remains obscure, it may be preferable to describe them as "acute gastric mucosal lesions" (AGML), clinically manifested by "acute gastric mucosal bleeding" (AGMB).

INCIDENCE

Only recently has the clinical importance of this condition been appreciated. Because gastric mucosal erosions cannot be demonstrated by the usual radiological diagnostic methods, radiologically negative upper gastrointestinal bleeding has all too often been attributed to "peptic ulceration." Only after routine diagnostic gastroscopy became practically feasible with the introduction of fiberoptic instrumentation in the early and middle sixties, did the importance of the condition under discussion become evident. Before fiberoptic gastroscopy became popular, upper gastrointestinal tract bleeding was attributed to "peptic ulceration" in 48–75 percent of cases and to AGML in less than 5 percent of cases. After early gastroscopic examination became a part of the routine diagnostic work-up of patients with upper gastrointestinal bleeding, it became obvious that AGML was responsible in at least 20 percent of cases (2). In the experience of this reviewer and of others (3), AGML are the most common cause of bleeding in patients hospitalized for upper gastrointestinal hemorrhage.

Because AGMB is often precipitated by one of several situations to be discussed below, the frequency with which these situations are encountered in a given institution will determine the incidence of AGML in that particular institution. In a recent autopsy study of burned patients, the incidence of AGML at autopsy was 28 percent (4). In a similar study of 1700 patients treated at the Brooke Army Medical Center, the incidence of acute gastroduodenal ulceration was 13 percent (5). Half of the latter had multiple gastric "ulcers." Over a recent 3-year period, 300 patients treated at the Detroit General Hospital for multiple organ injury (excluding patients with AGMB related to alcohol, drugs, etc.) developed AGML requiring transfusions (3).

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PATHOLOGY

Gross.—The gastric mucosal membrane of patients with AGMB is characterized by the presence of multiple erosions ranging in size from a pinpoint to 1 cm in diameter. Occasionally one may encounter huge lesions involving the entire aspect of one wall of the stomach or the other. The distribution of the lesions may vary with the condition which has precipitated the AGMB. When the onset of AGMB has been preceded by the "stress" of a major operation or severe trauma, the lesions tend to cluster in the proximal portion of the stomach on the greater curvature and spread toward the junction of the body of the antrum (3). AGML complicating the ingestion of such ulcerogenic drugs as aspirin are more often found on the lesser curvature of the stomach. By contrast, the lesions occurring in patients having sustained major thermal burns frequently involve the distal portion of the stomach and may be accompanied by single or multiple duodenal ulcers (6). The mucosa may be covered by innumerable petechiae giving the impression of a diffuse weeping of blood from the mucosal membrane or the mucosa appears to be diffusely infiltrated with blood (Fig. 1). Or, one may find at operation that the mucosal surface is covered with numerous small clots. When these are wiped off, minute erosions are revealed. When operating on patients with this condition, one is impressed by the "friability" of the gastric mucosal membrane which bleeds readily upon contact with an instrument or sponge. One of the most characteristic features of AGML, in this reviewer's opinion, is the finding at operation or at autopsy of mucosal lesions in various stages of evolution: lesions in the stage of hemorrhagic mucosal necrosis side by side with others where the necrotic mucosa already sloughed and the erosion has entered a phase of healing (Fig. 2).

Microscopic.—The characteristic pathologic feature of AGMB is the gastric mucosal erosion (Fig. 3). (It is important to distinguish an erosion from an ulceration. An erosion is a lesion extending no deeper than the muscularis mucosae. Any lesion extending deeper than the latter structure becomes, by definition, an ulceration.) The striking microscopic finding in this condition is the presence of focal mucosal hemorrhage leading to hemorrhagic and coagulative necrossis (Fig. 4). The subsequent sloughing of these foci of hemorrhagic necrossis leaves the characteristic erosions (Fig. 3). Elsewhere, one may find diffuse mucosal hemorrhage, edema and hemorrhage in the submucosa, with congestion of the submucosal vessels (Fig. 5). In the membrana propria, mucosal edema under the surface epithelium and around the foveolae may also be present. Often (7), one finds that the surface epithelium is absent and appears to have sloughed at the level of the neck stratum of the gastric glands.

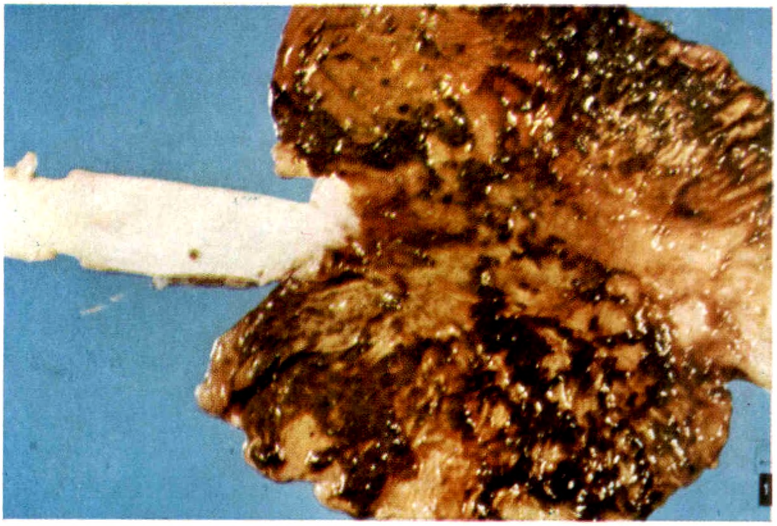

FIGURE 1. Exsanguinating hemorrhage from AGML in a patient receiving large doses of steroids.

When AGMB has been precipitated by burns, particularly when the latter have been complicated by sepsis, frank ulceration or ulcerations may also be present in the stomach or in the duodenum (4–6).

A recently recognized feature of the AGML, particularly those associated with sepsis complicating severe stress and burns (6, 8), is the presence of stainable microorganisms on the surface of the craters and erosions and in blood clots overlying the gastric mucosal membrane (Fig. 6).

One aspect of this condition which has impressed the reviewer is the frequency with which the erosions are so minute that they can be seen only with the aid of the microscope. When a stomach contains only lesions of this nature and when the bleeding has ceased at the time of laparotomy, as it often does, one may have the false impression upon opening the stomach and after wiping out the blood and clots, that the gastric mucosal membrane is normal. This situation may be one of the rare indications for a "blind" resection as long as one is certain that the hemorrhage is coming from the stomach.

Associated with the acute changes described above and which are responsible for the bleeding, one may find varying degrees of chronic gastritis (9, 10). Often these changes are focal and vary in severity from one portion of the segment to another: thickening of the muscularis mucosae, thinning of the mucosal membrane, decreased number of gastric glands, nodular lymphoid hyperplasia, cystic dilatation of foveolae, intestinal metaplasia, and diffuse round cell infiltration in the membrana propria (Fig. 7). Sometimes these chronic changes are so severe as to suggest that the patient's stomach was incapable of secreting any appreciable amounts of HCl. Indeed,

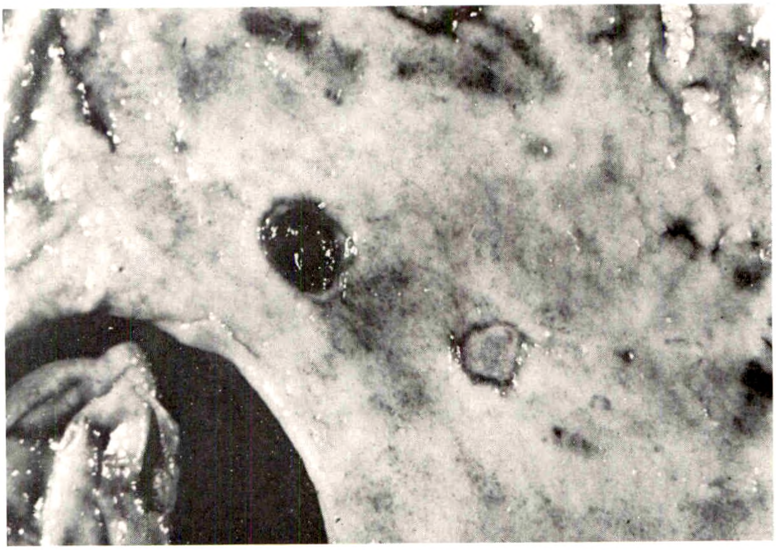

FIGURE 2. Aspirin-induced gastric erosions. Note the different stages of evolution of the 2 lesions.

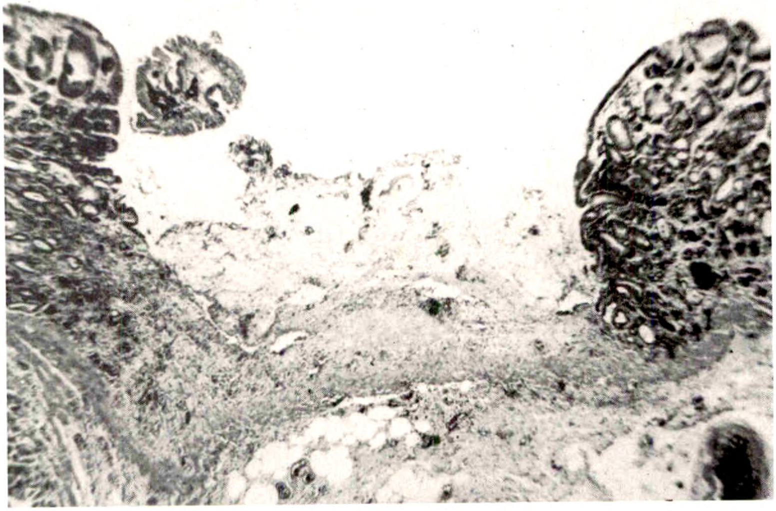

FIGURE 3. Low power view of an erosion. Note that the base of the lesion does not extend beyond the muscularis mucosae.

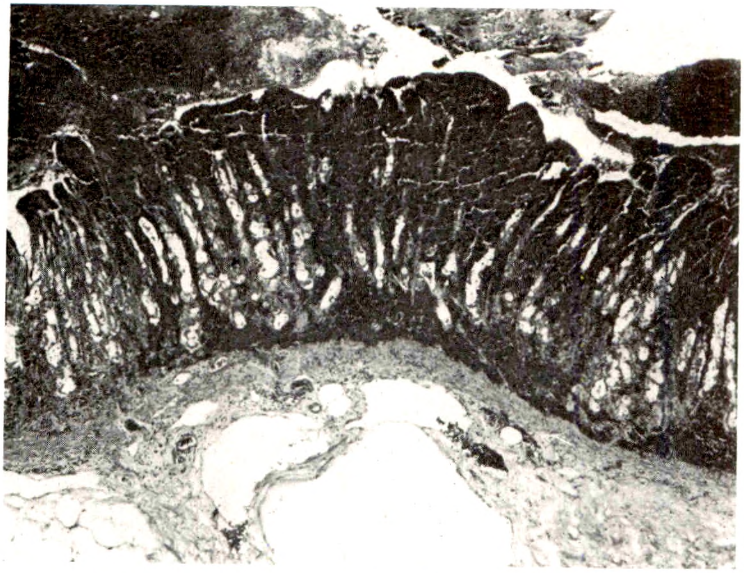

FIGURE 4. Focal hemorrhagic and coagulative necrosis.

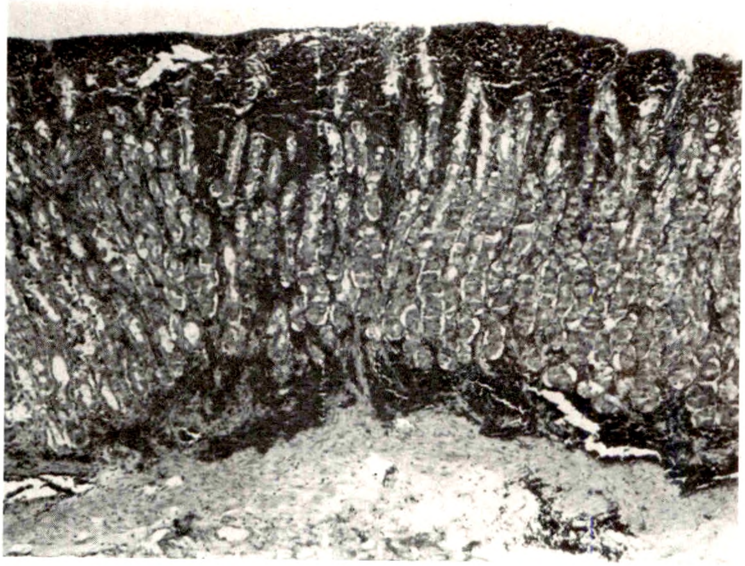

FIGURE 5. Diffuse mucosal and submucosal hemorrhage.

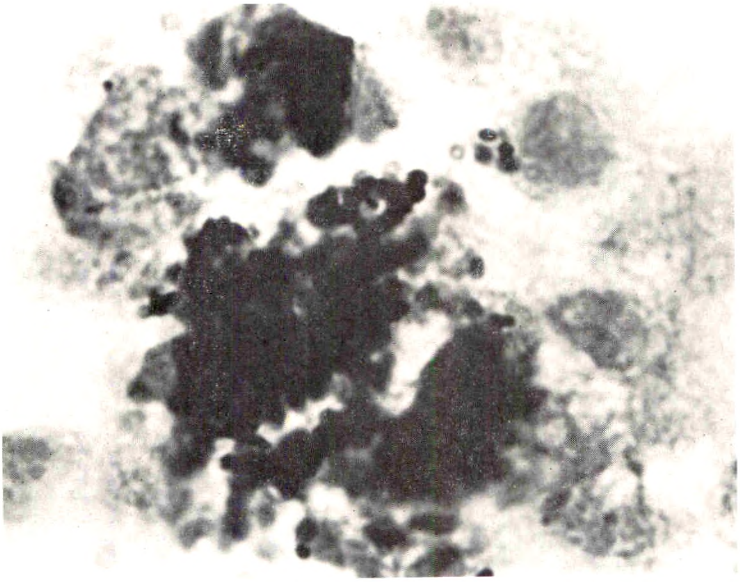

FIGURE 6. Bacterial colony on the gastric mucosal surface of a patient with stress-induced AGMB.

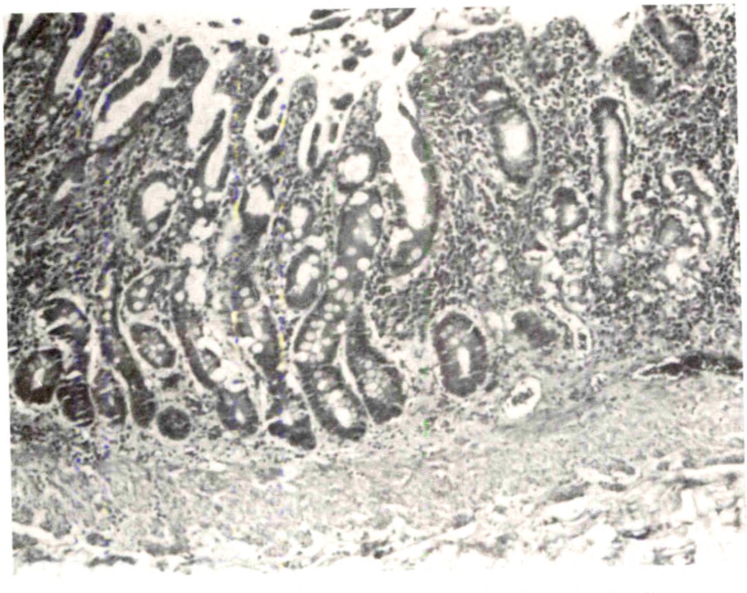

FIGURE 7. Changes characteristic of chronic gastritis in the fundic mucosa of a patient with massive, spontaneous AGMB.

in two cases encountered by the author, the chronic gastritis accompanying the AGML had been associated with a previously diagnosed and treated pernicious anemia. On the basis of the presence or absence of associated chronic gastritis, Menguy et al have separated patients with acute gastric mucosal bleeding into two categories: those with and those without antecedent chronic gastritis (11).

ETIOLOGY

The intimate mechanism(s) leading to AGML remain unknown. However, it has become gradually recognized that AGML is often preceded by one of several situations characterized either by stress (11, 12), the administration of one of several drugs (13) all of which share the common property of having anti-inflammatory effects, and alcohol (13, 14). More recently, the spontaneous occurrence of AGMB without one of the antecedent situations alluded to above has been recognized more often (9). Menguy et al (11) recently reviewed a series of patients who required surgery for the control of massive bleeding due to AGML. The stomachs of those patients whose AGMB was not associated with an antecedent history of "ulcerogenic drugs," alcohol, or stress, all showed changes of moderate-to-severe atrophic gastritis. On the other hand, the stomachs of the patients whose bleeding appeared to have been precipitated by one of the usual "causes" of AGML only showed the acute changes responsible for the bleeding. The relationship of these various situations to AGML is summarized in Figure 8.

Of singular importance in the development of AGML following such situations as severe trauma or burns, is the development of septic complications of the latter. Pruitt (6) noted that in patients with burns involving less than 50 percent of their body surface, only 2 percent of those without septic complications developed AGML, whereas the incidence of the latter in patients with sepsis was 19 percent. Skillman (15) has also drawn attention to the importance of sepsis in this condition.

In this review, we may appear to be considering as a discrete entity a situation characterized by the appearance of superficial erosions in the gastric mucosal membrane. This approach may or may not be valid. It is likely, even probable, that the intimate mechanisms leading to the formation of gastric mucosal erosions in patients with thermal burns complicated by sepsis differ from those operating after major trauma or the ingestion of aspirin. There are some indications that such differences may indeed exist. For instance, AGML complicating thermal burns tend to involve the distal stomach and are often accompanied by acute duodenal ulcerations (5, 6). Moreover, the lesions complicating burns often perforate. By contrast, acute lesions complicating the administration of anti-inflammatory drugs or trauma often involve more proximal segments of the stomach and bleed far more often than perforate (3, 11).

However, even though we have not yet learned to distinguish the various mechanisms leading to different types of AGML, at the very least, we have learned to differentiate AGML from gastric and duodenal ulcers.

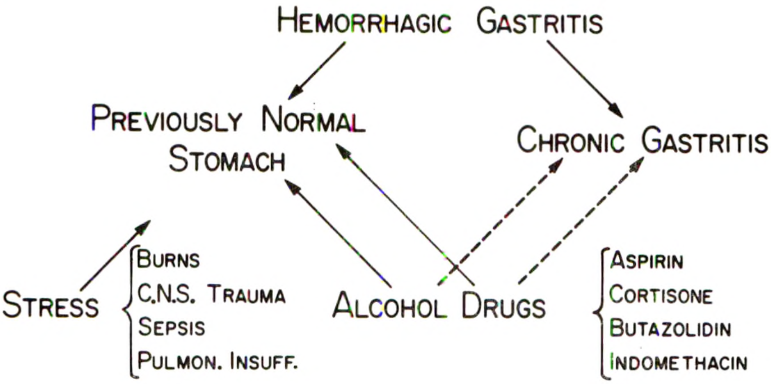

FIGURE 8. Situations commonly associated with AGMB.

MECHANISMS OF FORMATION OF AGML

As previously stated, the mechanisms leading to the formation of erosions and bleeding from the gastric mucosal membrane are not known. The two mechanisms which have received the most interest recently are gastric hypersecretion and abnormalities in the gastric mucosal barrier.

Gastric hypersecretion.—Because of the well-known association between duodenal ulceration and gastric hyperacidity, it has been assumed that gastric hyperacidity, in addition to causing duodenal ulceration, may also be responsible for gastric erosions. However, the evidence for such a relationship is, at best, tenuous. With respect to the mucosal erosions complicating the administration of such anti-inflammatory drugs as aspirin (16), cortisone (17), phenylbutazone (18), and indomethacin (19), the role of gastric HCl has never been proven. With the exception of cortisone and related compounds which, under certain conditions and in very large doses may augment gastric acidity (20), these drugs have very little influence on gastric secretion of acid. The strongest argument for a relationship between AGML and gastric acidity may be found in observations of increased levels of gastric acid in patients having undergone neurosurgical procedures (15) which are known to be occasionally complicated by AGMB (21). On the other hand, no one has been able to demonstrate a consistent pattern of hypersecretion of acid in patients having sustained thermal burns (5, 6). Moreover, stainable microorganisms on the surface of the gastric mucosal membrane have been found in burned patients (6, 8), a finding which is not consistent with gastric hyperacidity. Mild acid hypersecretion has been measured in patients with AGMB complicating trauma (3). It has been pointed out that because an abnormally permeable gastric mucosal barrier may be the salient gastric mucosal abnormality precipitated by "stress," the inability to measure gastric hyperacidity in stressed patients may be due to an abnormally high degree of back-diffusion of hydrogen ion through an abnormally permeable gastric mucosal membrane (22). In other words, hydrogen ion may be secreted in higher concentrations, but may not be measured, i.e., collected, because it is escaping through the barrier. A corollary of this view would be that an abnormally permeable mucosal membrane may thus be more readily injured by subnormal concentrations of HCl in the stomach.

One argument against the importance of gastric hyperacidity in all cases of AGML is the observation that in certain types of AGML, particularly those that are not associated with such precipitating factors as burns, stress, or drugs, there is an underlying chronic gastritis (9–11), often very severe and sometimes associated with pernicious anemia, which indicates that the stomach was incapable of secreting appreciable amounts of acid even though measurements of HCl may not have been made prior to the bleeding. Since severe, chronic gastritis appears to be conducive to the development of AGML, a brief consideration of some of the factors underlying its development may be worthwhile. Such factors as alcohol and particularly the combination of the chronic ingestion of alcohol and aspirin have received increasing attention recently (14). Of great interest also in recent years has been the observation that chronic gastritis and/or chronic gastric ulcer is often associated with an abnormal degree of reflux of duodenal contents into the stomach (23). It has been shown experimentally that the prolonged exposure of the gastric mucosa to bile produces in the gastric mucosal membrane of dogs, mucosal changes very similar to those of spontaneously occurring chronic gastritis in humans (24).

Abnormalities in the gastric mucosal barrier.—Hydrogen ion in gastric juice is separated from the mucosal cells by: (*a*) the site of secretion of HCl; (*b*) the glandular tubule through which the secretion flows; (*c*) the mucosal surface consisting of a single layer of cells covered by a layer of mucus. Under normal conditions, the permeability of this gastric mucosal membrane to hydrogen ion is small. When the gastric mucosa is injured by any one of a variety of agents such as eugenol, acetic acid or aspirin, or alcohol, the mucosal permeability to hydrogen ion is increased, allowing it to diffuse back into the mucosa with resulting injury to its structure and bleeding. Much of the experimental work supporting this view has been carried out by Davenport (25–29).

Do the clinical or experimental conditions commonly associated with the development of AGMB affect the permeability of the gastric mucosal membrane? Experimental studies in rabbits have shown that hemorrhagic shock produces gastric mucosal vasoconstriction and erosions (30). Also, significant back-diffusion of HCl can be demonstrated within 3 hours after the induction of hemorrhagic hypotension (22). Other studies have indicated that a similar abnormal gastric mucosal permeability to hydrogen ion exists in critically ill patients (22). It has been suggested that the gastric hypoacidity often found in critically ill patients may result from this loss of hydrogen ion via back-diffusion rather than from an absolute decrease in gastric secretory activity (22).

Regarding the mechanisms via which stress and some of the other situations alluded to previously may alter the permeability of the gastric mucosal membrane, only very few experimental studies have been carried out. Menguy and associates have suggested that AGML following stress and/or the administration of adrenal cortical steroids may result from steroid-induced changes in the mucus and cellular components of the gastric mucosal barrier. They found that the administration of cortisone or ACTH to dogs reduced the rate of mucous secretion (17, 31) and altered the composition of gastric-antral mucus in a way that rendered the mucosubstance more readily digestible by pepsin and trypsin (32). Also, they found that these same compounds reduced the rate of cell turnover in the canine stomach, a change which can be expected to affect adversely the ability of the mucosa to maintain its integrity (33). Other workers have found that the induction of hemorrhagic shock in dogs produces acute gastric erosions preventable by pyloric ligation or the administration of trypsin inhibitors (34). On the basis of this work, it was postulated that the reflux of duodenal secretions into the stomach during shock may injure the gastric mucosal barrier.

With respect to the problem of gastric mucosal injury by aspirin, Davenport has suggested that it is the direct contact of aspirin with the gastric epithelial cells that leads to their injury. He found that when he irrigated canine gastric pouches with aspirin in acid solutions, gastric mucosal damage with fluid and ionic diffusion into the lumen of the pouch resulted (27). He theorized that acid conditions in the stomach favored the absorption of the un-ionized fat-soluble form of aspirin across the gastric mucosal barrier with resulting injury of the gastric mucosa and an increased permeability to hydrogen ion which in turn would cause the mucosal damage resulting in bleeding. Although it is probable that at least part of the aspirin-induced gastric mucosal injury results from a direct contact of aspirin with the gastric mucosa, it is also true that in man as well as in experimental animals, gastric erosions occur following the parenteral administration of aspirin (16). Menguy and associates have reported that the parenteral administration of aspirin to rats or the oral administration of aspirin to dogs with antral pouches or Heidenhain pouches reduced gastric secretion of mucus (35) and increased the rate of cell loss from the gastric mucosal surface (33), loss which was not accompanied by an increased rate of cell turnover. On the basis of these experiments, they theorized that circulating aspirin may also play a role in altering the permeability of the gastric mucosal barrier to hydrogen ion.

Clinical Manifestations

On the surface, it would appear to be redundant to point out that the salient clinical manifestation of AGML is bleeding. However, there are some clinical features of this condition that are worth discussing. Firstly, it is important to realize that massive bleeding may not become clinically manifest and the underlying condition may be discovered only at autopsy. Such was the case in approximately 1/4 of patients with AGML complicating thermal burns (6). Therefore, one should be on the alert for such signs of occult bleeding as a rapid pulse and a reduced blood pressure in patients suffering from sepsis complicating severe burns or trauma, a situation conducive to the development of AGML. It is important to have some idea of the average interval between the development of the conditions conducive to the formation of AGML and the onset of gastric mucosal bleeding. Because sepsis complicating burns or severe trauma appears to be such an important factor in precipitating AGMB, it may be more useful to estimate the interval between the appearance of sepsis and the onset of bleeding. The average interval in 3 different series of cases was 9 days (15), 3 days (3), and 5 days (6), respectively. Flowers et al found that AGMB complicating surgery occurred most often between the seventh and tenth postoperative days (36).

A striking feature of the bleeding in our experience is its intermittent nature, sometimes over many days. This contrasts sharply with the bleeding from peptic ulcer which usually stops completely when conservative treatment has been successful. On the basis of pathologic specimens, this reviewer has suggested that the intermittent nature of the bleeding may be due to the formation of fresh erosions as earlier ones heal (11). It is also possible that further bleeding may in some cases be due to mucosal injury by a naso-gastric tube. It has also been this reviewer's impression that this intermittent bleeding is particularly characteristic of patients with an underlying atrophic gastritis. When the AGMB complicates a severe illness or multiple organ injuries, it is often overshadowed by the underlying illness. Particularly in patients with thermal burns, one should anticipate the possibility of a perforation, the manifestations of which may be completely masked by signs and symptoms of the primary illness and by the upper gastrointestinal bleeding.

MEDICAL MANAGEMENT

Obviously, the most important feature of nonoperative management of AGMB as for any other condition associated with blood loss is blood replacement. Obviously, hypovolemia and inadequate tissue perfusion represent an additional stressful factor which may be intolerable for a patient already desperately ill. The blood replacement must be aggressive and yet accurate and careful with extreme attention to the patient's cardiac reserve which is often marginal under the circumstances surrounding AGMB.

Gastric cooling remains the cornerstone of medical management. During the early sixties, there was a wave of interest in the use of cooling machines for the treatment of gastro-duodenal hemorrhage. Although the value of gastric cooling in the management of bleeding from duodenal or gastric ulcer or esophageal varices has never been substantiated, it may be beneficial in the management of AGMB. However, the use of mechanically refrigerated cooling machines has gradually been abandoned in favor of cooling the gastric mucosa with iced saline. The latter has the added advantage of removing blood from the stomach which, in addition to facilitating immediate endoscopy, may also favor gastric mucosal hemostatsis by allowing the stomach to contract. Also, Lucas et al have drawn attention to the prognostic value of the response to iced saline lavage. They noticed that the patients who stopped bleeding with this treatment, even if they rebled later, had a better prognosis than those who showed no response at all (3).

In some instances, it may be possible to achieve nonoperative gastric mucosal hemostasis by opening up mucosal A–V shunts and reducing gastric mucosal blood flow. Although vasopressin has been used for this purpose for a long time, the results have been, at best, questionable. It is possible that more recent methods of selective superior mesenteric infusions of vasopressin may be beneficial (37).

One should not leave the area of medical management of AGMB without discussing the advantages and disadvantages of naso-gastric intubation. In the presence of upper gastrointestinal bleeding, the use of a naso-gastric tube aids in the evaluation of the severity of the hemorrhage, response to management, and is necessary for gastric lavage, gastric emptying and prevention of aspiration pneumonitis. However, it is also evident that in some cases the presence of the tube, particularly when attached to strong suction, may further injure an already fragile gastric mucosal membrane. It is therefore important to withdraw the naso-gastric tube when its continued presence in the stomach is no longer absolutely necessary.

SURGICAL MANAGEMENT

The surgery of AGMB remains controversial. Historically, surgeons have applied to the management of "hemorrhagic gastritis" the operation most commonly used for the treatment of "peptic ulcer" at the time. Through the forties and the fifties, partial gastrectomy was most often applied to this condition and several reports have emphasized the frequency of rebleeding after partial gastrectomy for hemorrhagic gastritis. Gilchrist in 1954 reported that all of 4 patients, 3 of whom died, rebled after a partial gastrectomy (38). In 1961, Rosenkrantz described rebleeding in 7 of 14 patients having undergone a partial gastrectomy (39). In 1959, Palmer reported that 3 of 4 patients treated by partial gastrectomy rebled and died (7). Because of the discouraging results following a partial gastrectomy, total gastrectomy has been advocated and performed from time to time. Spencer & Maloney suggested that the failure of partial gastrectomy to control bleeding from AGML could be due to mucosal lesions remaining in the gastric pouch, and they recommended a wide gastrotomy to establish the level of resection (40). On the other hand, Menguy et al have pointed out that although no mucosal lesions may be present in the gastric pouch remaining after a partial gastrectomy, fresh lesions may appear subsequently in the remaining stomach and cause rebleeding (11).

In 1964, the report of Sullivan et al (41) describing complete remission from bleeding from AGML after a vagotomy and pyloroplasty seemed to represent a very real progress in the surgical treatment of this condition. The possible beneficial influence of vagotomy for AGMB was attributed to opening of mucosal A–V shunts rather than to reduction in acid secretion (41). However, in a subsequent report from the same group, a higher incidence of postoperative rebleeding was experienced (42). The experience of other surgeons with this approach has been less satisfactory. Nagel reported rebleeding in 2 of 14 patients with AGML treated by vagotomy and pyloroplasty (43). Bryant & Griffen described rebleeding in 4 of 5 patients with "stress ulcer" treated by vagotomy and pyloroplasty (44). Five of 9 patients reported by Goodman & Frey rebled after a vagotomy and drainage procedure (45). Carruthers reported rebleeding in 3 of 9 patients with "erosive gastritis" treated by vagotomy and pyloroplasty and suture of bleeding points (46). Menguy et al (11) have suggested that when AGMB is severe enough to require surgery, the method of treatment should be tailored to the individual patient. When the patient is so seriously ill, usually because of the condition that precipitated AGMB (severe burn or multiple organ injuries, etc), the operation to be performed should be one that guarantees against further bleeding, i.e. a near-total gastrectomy. On the other hand, if the patient's condition is such that rebleeding after a more conservative procedure would not be prejudicial to his survival, a vagotomy and antrectomy or vagotomy with pyloroplasty and suture-ligation of all visible bleeding points should be carried out. In other words: the worse the risk, the more radical the procedure. This point is a crucial one for, as Kirtley et al (47) have stressed recently, rebleeding after operation for AGMB carries a very high mortality. None of the therapeutic modalities introduced recently in the surgery of this condition such as vagotomy and pyloroplasty warrant, in our view, a change in the position stated so clearly by Palmer (7), an early student of the problem:

> "The important error made in the emergency surgical approach was that of being content with less than total resection of the stomach, in spite of the fact that gastroscopic examination had shown the erosive process to be universal. . . . Except for the dictation of experience, there is much, of course, to discourage radical, total gastrectomy: often the patient has never had any stomach troubles before the sudden hemorrhage. Erosive gastritis is an acute process which may never recur and the patient may never have stomach trouble again if he survives the current episode. Sometimes the bleeding stops spontaneously in the proximal stomach remnant during or following subtotal resection or at least this can be hoped for. *It is not easy to learn the hard lesson of radical finality under these circumstances until a few errors, such as those reported here, have been made.*" (Fig. 9).

The results of various operations commonly applied to the management of acute gastric mucosal bleeding are summarized in Table 1. A point not often sufficiently stressed is that mortality from AGMB treated medically or surgically may be due either to the primary condition compounded by sepsis or to the bleeding, or to the aggravation of the former by the latter. For this reason, the evaluation of the efficacy of any one of the therapeutic

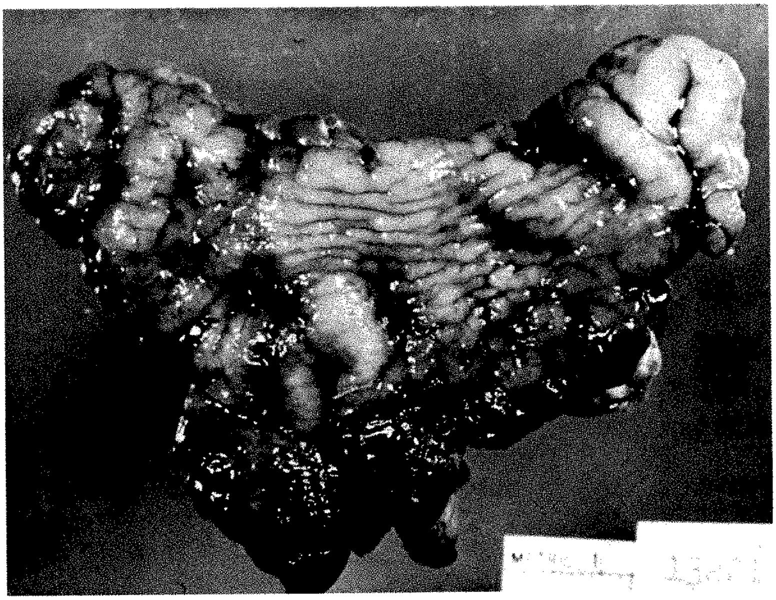

FIGURE 9. This 64-year-old man underwent a 90% gastrectomy (author) for massive AGMB (stress+aspirin). A week later he rebled massively (22 units over 48 hr) from this small gastric remnant. He expired 4 weeks later from complications related to the second operation.

TABLE 1. RESULTS OF OPERATIONS USED IN THE TREATMENT OF AGMB

	Total gastrectomy		Partial gastrectomy		Partial gastrectomy and vagotomy		Vagotomy and pyloroplasty	
	No.	% Rebled	No.	% Rebled	No.	% Rebled	No.	% Rebled
Palmer (7)	1	0	4	75	—	—	—	—
Rosenkrantz et al (39)	1	0	14	50	—	—	—	—
Ferguson et al (48)	—	—	—	—	—	—	9	33
Sullivan et al (41)	—	—	—	—	2	0	8	0
Sullivan et al (42)	—	—	—	—	—	—	28	14
Bryant et al (44)	—	—	—	—	—	—	5	80
Olsen et al (49)	—	—	—	—	1	100	14	50
Goodman et al (45)	—	—	—	—	—	—	9	55
Nagel et al (43)	—	—	—	—	2	100	12	17
Foster et al (50)	—	—	—	—	4	50	14	14
Carruthers et al (46)	—	—	—	—	—	—	9	33
Lulu et al (51)	—	—	4	50	12	8	3	100
Menguy et al (11)	10	0	16	50	6	50	4	100
Kirtley et al (47)	—	—	—	—	7	14	26	15
David et al (52)	—	—	7	14	6	33	20	55

modalities previously mentioned on the basis of absence or presence of rebleeding seems more useful than judging it on the basis of patient survival. For instance, of 323 cases of AGML complicating thermal burns, 69 percent died. However, only 19 percent of the patients died from causes related to AGML (6). The others died of sepsis. The lethality of the combination of circumstances leading to the development of AGMB is also emphasized by the report of Skillman et al who noted that of 8 patients out of 150 patients treated in their respiratory intensive care unit who developed AGMB, only one survived (15).

AGMB is being recognized as one of the most common causes of upper gastrointestinal bleeding. Because of the circumstances which surround its occurrence, it is a highly lethal problem. With the gradual aging of the patient population, with the increased prevalence of trauma and with the growing complexity of surgical operations, it is likely that this problem will continue to increase in frequency.

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CHOICE OF OPERATIONS IN THE MANAGEMENT OF SYMPTOMATIC SLIDING HIATAL HERNIA[1] 7044

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The true incidence of esophageal hiatal hernia remains unknown. It has been said to be present in as many as 20 percent of upper gastrointestinal series. Since the latter are rarely completely "routine," such figures are meaningless. The frequency with which a hiatal hernia causes symptoms is indicated by the data of Boyd (1) who found that 18 percent of patients with a hiatal hernia remained asymptomatic, 7 percent developed complications, and 4 percent underwent surgical corrections of their hernia. As pointed out by Rex et al (2), nonoperative management in the face of symptoms of reflux esophagitis fails more often than when it is applied to the treatment of asymptomatic patients or those with only minor symptoms. Little controversy surrounds the principles upon which the medical treatment of hiatal hernia is based. Unfortunately, the same is not true when it comes to considering an operative correction. The bulk of the literature containing the operative results of hiatal hernia repair has been written by surgeons (as this review has been) and the reporting of "new" operations often has confused enthusiasm with scientific objectivity. All this may have left the nonoperating clinician wondering whether or not his surgical colleagues know what they are doing, a state of confusion well expressed by Pope's plaintive query, "Faced with a patient with symptomatic or anatomical complications of esophageal reflux, what is a clinician to do?" (3). The purpose of this review is to examine the worth of the various operations which have been applied to the management of complicated sliding hiatal hernia.

PRE- AND POSTOPERATIVE EVALUATION OF PATIENTS WITH HIATAL HERNIA

A defect common to many reports dealing with surgical correction of a hiatal hernia is an inadequate or complete lack of objective preoperative and postoperative evaluation of reflux and of the severity of the esophagitis causing the patient's symptoms.

Measurements of esophageal motility have been found helpful by some in recognizing small hiatal herniae on the basis of abnormal motor patterns in the zone of the lower esophageal sphincter (4). However, this test lacks accuracy and none of the motor abnormalities that has been associated with hiatal herniation is common to all patients with this condition. Moreover, the equipment necessary for the proper measurement of esophageal motility as well as the technical personnel to operate it are not always available "in the field" where the bulk of this type of surgery takes place.

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The detection of esophageal reflux by the measurement of esophageal pH via a pH probe withdrawn from the stomach into the esophagus remains a valuable and simple test for detecting esophageal reflux (5). Unfortunately, the test suffers from a high incidence of false positive results unless one takes the precaution of recording esophageal pH in an antegrade manner (6). Of course, the test does not permit one to quantitate the degree of reflux.

Radiographic studies using cinefluorography have been found most helpful by others (7). This approach is valuable as long as the operator is experienced in proper positioning of the patient and application of abdominal pressure. Under these conditions, it is probably the most useful one for the average clinician as well as for the one who manages large numbers of patients with hiatal herniation. Radiography should be combined with esophagoscopy to evaluate the severity of esophagitis, which is almost always present to some degree in patients with symptoms of esophageal reflux. With the advent of the fibroscope, esophagoscopy has become a more accurate, easier and safer procedure than was the case with the older, rigid instruments. It is essential that at least the last two methods of evaluation be applied preoperatively, and at regular intervals postoperatively to all patients undergoing curative surgery. Nothing can be more misleading than relying on the patient's satisfaction in evaluating the worth of an operation.

SURGICAL OBJECTIVES IN THE MANAGEMENT OF SLIDING HIATAL HERNIA

The relative importance of the anatomic elements believed to contribute to the competency of the normal gastro-esophageal sphincter mechanism remains controversial. However, there is little disagreement on the major role of the intrinsic musculature of the lower 2–4 cm of the esophagus which constitutes the lower esophageal sphincter. The importance of other elements such as redundant gastric mucosal folds (8) at the gastro-esophageal junction, gastric sling fibers, the oblique entry of the esophagus into the stomach, if any, remain unproven. The main indication for surgery in these patients is esophageal reflux and therefore the main goal of surgery is the prevention of reflux. This may be achieved by replacing the lower esophagus in an intra-abdominal position where it is thought to function more effectively or reinforcing the lower esophageal sphincter by one of several techniques to be described below.

TECHNIQUES BASED ON RESTORATION OF THE LOWER ESOPHAGUS TO AN INTRA-ABDOMINAL POSITION

One of the most widely used surgical approaches to the management of sliding hiatal hernia is the Allison or crural repair (9). This method empha-

TABLE 1. **Results of Allison Procedure for Esophageal Hiatal Hernia**

Author	Recurrence	
	Clinical	Radiological
Allison (9)	—	6%
Sparks (10)	—	10%
Brintnall et al (11)	42%	48%
Borgeskov et al (12)	40%	34%
Edwards et al (13)	20%	62%
Raphael et al (14)	14%	25%
Pearson et al (15)	17%	60%

sizes reduction of the hernia, restoration, and maintenance of normal anatomy through approximation of the diaphragmatic crura. Because of the high anatomic and symptomatic recurrence rate attached to this operation (Table 1), its popularity is rapidly declining. Nevertheless, the rationale upon which the Allison repair is based, namely, improvement of the function of the lower esophageal sphincter through restoration of normal anatomy, remains valid. In most cases, replacement of the gastro-esophageal junction in its normal intra-abdominal position provides the patient with complete symptomatic relief. The only problem with the procedure is that all too often, the repair fails to hold and the cardia slides back once again into the posterior mediastinum, with recurrence of the symptoms of esophageal reflux. Recognition of the very high incidence of symptomatic and anatomic recurrences after the crural repair led to the introduction in the fifties of ancillary procedures designed either to prevent an anatomic recurrence or to minimize its consequences. Some have attempted to reinforce the crural repair with fascia lata (16). Naturally, when synthetics such as Teflon mesh were introduced into the armamentarium of hernia repair in other areas, the use of these materials was applied to the reinforcement of crural repair (17). A most unfortunate practice and one which was widely applied at one time was the placement of sutures between the fundus of the stomach and the undersurface of the diaphragm in the hope of anchoring the stomach below the diaphragm and preventing its dislocation into the posterior mediastinum. As Hill (18) pointed out, this type of suturing may indeed prevent a recurrence of the "hernia," but by splinting the gastro-esophageal junction it may also prevent the lower esophageal sphincter from closing properly and thus aggravate the reflux.

Others have attempted to minimize the consequences of an anatomic recurrence by combining a crural repair of the hernia with procedures such as a vagotomy and pyloroplasty (19) or a partial gastrectomy with a Billroth I or II anastomosis (20, 21), operations designed to reduce gastric acidity. These operations were based upon the premise that the symptoms of hiatal herniation and the severity of the attendant esophagitis correlate with the level of the patient's secretory activity. By reducing the latter via a "duodenal ulcer operation," one would hope to minimize the consequences of esophageal reflux should an anatomic recurrence follow the attempted surgical reduction of the hernia. This approach, popular throughout the late fifties and early sixties, has been abandoned by most surgeons with a broad experience in the management of hiatal herniation because of several deficiencies. Firstly, it is based on the expectation that the important part of the operation may fail, an unfortunate attitude vis-a-vis any operation. It may leave the patient with some of the undesirable sequellae of a vagotomy and loss of the reservoir function of the stomach (22). A more damning argument against the use of "ulcer operations' for the correction of symptoms of esophageal reflux can be made on the strength of recent data indicating that the gastric antral hormone, gastrin, increases the strength of the lower esophageal sphincter (23). Reducing the ability of antral mucosa to produce gastrin by vagal denervation of the stomach, or removing the source of gastrin by distal gastric resection should effect adversely the function of the lower esophageal sphincter as a barrier to esophageal reflux of gastric contents. Moreover, the severity of esophagitis does not correlate with the patient's level of gastric acidity (24–26), to which observation one may add the now well-documented fact that esophageal reflux of *alkaline* duodenal contents may also cause a severe esophagitis (27, 28). Because the destruction of the pyloric sphincter mechanism by a drainage procedure or distal gastrectomy increases the likelihood of alkaline reflux into the stomach, reflux which may be favored by a vagotomy causing gastric stasis, it appears that these ulcer operations done in conjunction with a hiatal hernia repair may leave the patient worse off than he was before the operation should the repair fail to hold, in addition to the danger of leaving the patient with such complications of these ulcer operations as postvagotomy diarrhea or the dumping syndrome. They should not be undertaken unless independently judged to be necessary for the management of coexisting duodenal ulcer.

Another approach to the surgical management of a hiatal hernia is also based on the premise that the lower esophageal sphincter functions better when it is situated in its normal intra-abdominal position. In crural repair, one attempts to achieve this goal by closing the esophageal hiatus around the esophagus to prevent the gastro-esophageal junction from sliding into the posterior mediastinum. By performing a *gastropexy* one attempts to prevent axial sliding by *anchoring* the stomach in the abdomen. Fixation of the lesser curvature of the stomach to the anterior abdominal wall has been recommended by some (29, 31). The results of this procedure are not satisfactory, probably because of the disparity in the planes of the anterior abdominal wall and the esophageal hiatus, and it has been abandoned by its main proponent (30).

Another form of gastropexy in which the phreno-esophageal membrane is sutured to the median arcuate ligament of the aortic hiatus has been applied extensively by Hill with apparently good results (32). Some of the success of the Hill posterior gastropexy has been attributed to the plication of the lesser curvature of the stomach around the distal esophagus so as to create a valve-like mechanism, not unlike the one created by fundoplication to be described below (32). Another type of posterior gastropexy in which the cardia is anchored to the origin of the diaphragm on the vertebral column in conjunction with crural closure anterior to the esophagus has been described by Collis (34). Although his results were good—9 failures in 82 cases—the method seems less satisfactory than the one described by Hill since it does not result in an exaggerated intra-abdominal segment of esophagus.

The rationale of both crural repair and gastropexy is the thesis that the lower esophageal sphincter functions more effectively in its normal intra-abdominal position. However, some clinical and physiologic data do not entirely support this thesis. Firstly, there is the well-known observation that many patients with a sliding hiatal hernia do not have reflux, a simple fact which clearly militates against the primary role of the "normal anatomy." Also, one may find evidence of gastro-esophageal reflux in patients without a demonstrable hiatal hernia (35). To these, one should add recent observations indicating that the pressure measured within the length of the lower esophageal sphincter is not as dependent upon the normal anatomic location of the sphincter as was formerly thought. Cohen & Harris found that the presence or absence of a sliding hiatal hernia does not affect the competence of the lower esophageal sphincter (36). (It should be pointed out that although these observations do not support the usual rationale for surgical reduction of a sliding hiatal hernia, one cannot discount the fact, too often documented to need re-emphasizing, that surgical repair of a sliding hiatal hernia *usually* corrects the symptoms of reflux. Dissatisfaction with this type of repair has not been due to the fact that it fails to work, but to its failure to hold.)

All of the procedures described above share a common theoretical drawback. They are not designed to correct the main reason for the symptoms associated with a sliding hiatal hernia: an incompetent lower esophageal sphincter. Recently, procedures designed to reinforce the sphincter mechanism have been applied extensively and it can be said that the surgery of esophageal hiatal herniation has entered a new era.

OPERATIONS DESIGNED TO REINFORCE THE LOWER ESOPHAGEAL SPHINCTER

During the past few years, several procedures designed to reinforce the lower esophageal sphincter and re-establish a long intra-abdominal esophageal segment have received increasing acceptance in this country after having been widely applied in Europe. The most popular of these procedures is the Nissen-Rossetti fundoplication first described in 1956 (37, 38). The procedure consists in the construction of a cuff of gastric fundus which is wrapped around the intra-abdominal esophagus. It is important that the sutures used to wrap the fundus around the esophagus also catch the latter, otherwise the fundoplication may become undone. In theory, the gastric cuff would transmit increased intragastric pressure to the esophagus, thus reinforcing the lower esophageal sphincter and preventing reflux. Experimental studies have supported the validity of this theory (33, 39, 40). Extensive experience with the operation in Europe (41–43) and in this country (44) indicates that it reliably prevents reflux, provides excellent symptomatic relief from the symptoms of esophagitis and has a low incidence of anatomic recurrences. Anatomic recurrence rates below 2 percent have been reported (43). Furthermore, an anatomic recurrence of herniation into the posterior mediatinum after a fundoplication may not have the same implication of symptomatic recurrence as it does with the crural repair, since fundoplication is effective in preventing reflux even when constructed in the chest in patients with acquired short esophagus and stricture (45, 46). It should be pointed out that occasionally patients, particularly women, who have undergone a Nissen fundoplication may experience mild postprandial bloating due to a reduced ability to eructate. Our patients have not found this to be a major problem as long as they are forewarned and instructed to avoid air-swallowing. In the hands of the occasional operator, a fundoplication may be a more hazardous procedure than a crural repair because of the technical problems in mobilizing the cardia from the spleen, which may be readily injured. Careless placement of the sutures in the wall of the esophagus may lead to serious septic complications should the sutures enter the esophageal lumen.

Another popular method of plicating the gastric fundus around the lower esophagus is the Belsey Mark IV procedure, a transthoracic operation. The long-term results of this operation as reported by Skinner & Belsey (47) are very good: 7 percent of patients had symptomatic or asymptomatic recurrences and 3 percent had persistent symptoms without herniation or detectable reflux. In our opinion, the operation is less appealing than the Nissen fundoplication because it requires a thoracic approach, whereas the latter can be done from above or below. Moreover, the important esophageal sutures in the Belsey procedure are placed in the long axis of the esophagus—in the line of the superficial, longitudinal muscle layer—and are more likely to tear out of the esophageal wall. A modification of the procedure has been described by Lind (48).

In an interesting study using the esophagus, stomach, and duodenum from autopsy human subjects, Butterfield (49) compared the ability of the Hill gastropexy and the Belsey and Nissen plication procedures to prevent esophageal reflux from the stomach filled with water under pressure. He found the Nissen and the Hill repairs to be the most successful in preventing reflux.

In summary, and at the risk of being accused of pleading pro suo domo, we wish to emphasize that real progress has been achieved during the past two decades in the surgical management of this common condition. Following a long period of evaluation of crural repair during which ancillary procedures based on attempts to reduce gastric acid secretion were added to the basic operation to improve its results, came the more physiologic era of the late fifties and sixties. Today we have in our armamentarium several sound procedures proven capable of reinforcing or replacing the action of the lower esophageal sphincter, the key element in the maintenance of a competent gastro-esophageal sphincter mechanism. It has been most gratifying to note that some of the traditional polemics surrounding the choice of approach—abdominal or thoracic, have lost their relevance.

If we may be permitted a personal note, our own preference in recent years has been for the Nissen fundoplication for severely complicated or recurrent lesions. We have found the Hill repair to be a very close second and a particularly valuable alternative when local anatomic conditions: small stomach, a previous gastrectomy, obesity rendering the construction of a fundoplication technically awkward.

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HEMODIALYSIS IN THE MANAGEMENT OF RENAL DISEASE

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In patients in whom a transplanted kidney is a lasting success, health and happiness may be completely restored. On the other hand, high doses of unspecific immunodepressant drugs aimed at producing tolerance to the graft, and high doses of corticosteroids may produce illnesses that are most cruel. It is to be wished that potential transplant recipients were adequately apprised of these dangers. The results of kidney transplantation have not improved as much as most of us had hoped. According to Simmons & Najarian (1), only 66 percent of unrelated (cadaver) kidney transplants have normal function after two years, and these results were obtained after recipients had been treated with antilymphocytic globulin, which indeed has improved the earlier results. Successful recipients presently run a risk (approaching 5 percent) of contracting a malignancy, usually reticulum cell sarcoma or lymphoma, but epitheliomas have also been seen. Following the work of Belzer (2), it is now possible to perfuse cadaveric kidneys and estimate their function more accurately. Perfusion also makes it possible to preserve the kidneys long enough to perform compatibility matching. The first statistically valid improved results accomplished by histocompatibility matching in unrelated donors, have just been reported at the meeting of the European Dialysis and Transplant Association in East Berlin (July 1971). Recurrence of glomerulonephritis in the transplanted kidney is still possible. This author believes that there is a place for dialysis in the treatment of chronic renal failure. Moreover, dialysis, even with the techniques we use now, will improve and will become an increasingly more acceptable alternative to transplantation. Every year there will be more patients needing treatment with artificial kidneys. The better treated they are, the more there will be the next year. Patients who are now being treated with transplants, if they do not die, will be coming to dialysis after three, five, or six years. It follows that the clientele treated by nephrologists will gradually change and that those who are sustained with artificial kidneys will gradually become a majority. The nephrologist who excludes himself from the use of dialysis will find himself in the same position as the colon surgeon who can remove only hemorrhoids; he will make money but will lost control of patients who are seriously ill. Peritoneal lavage has been misused the world over. Consequently, its reputation has suffered. McDonald showed that particulate matter which commonly occurs in intravenous fluids becomes important when 48 liters of peritoneal fluid are strained per 24 hours through the peritoneal cavity. Chemical irritation takes place around particles of glass or rubber. Tenckhoff (3) has really restored peritoneal lavage as a method of treatment for chronic dialysis by insisting on a closed system and absolute sterility (4). Present equipment is sterilized by heat. It is conceivable that reverse osmosis will be the method by which, in the future, peritoneal lavage fluid will be both sterilized and purified. The system developed in Kampen in the Netherlands in 1946 must be remembered (published by Kop and Kolff 5, 6). A single tank with two compartments would fit on the kitchen stove for sterilization. The fluid was mixed after cooling and would deliver 28 liters for peritoneal lavage, just enough to serve a patient overnight.

DIALYZERS

LARGE, FLAT-PLATE DIALYZERS

Large, flat-plate dialyzers, the most popular being the Kiil, have now been perfected so that their filling volume is reduced and their efficiency increased. The best Kiil is the Maxi-Kiil. Gotch (7), who has developed the technique of evaluating dialyzers to an unequaled level, has shown that the old Kiils varied greatly in individual performance, whereas the new ones are extremely reproducible. However, the trend is presently away from the Kiil. They are too large, too cumbersome, and too difficult to set up.

SMALL FLAT-PLATE DIALYZERS

Small, flat-plate dialyzers have a promising future, and both the manufacturers of coils and the manufacturers of capillary kidneys must remember that the smallest blood volume per surface area can be accommodated between parallel plates. (A sphere would be the largest blood volume per surface area.)

The most promising small flat-plate artificial kidney is not made in the United States, but is probably the one being developed by Holtzenbein in Germany. The time will come when very small flat-plate dialyzers will become available inexpensively and will be disposable. However, we cannot subdivide ad infinitum the flow between numbers of parallel plates as the flow may become so sluggish that sedimentation replaces cells and proteins (sedimentation is used for lack of a better word), and this was found both by Lavender (8) and Muir (9).

COIL KIDNEYS

Coil kidneys (10) are now available in variety. Their blood retention is getting smaller. Regenerated cellulose tubing, usually called cellophane, is still commonly used for dialysis. Specially designed tubes for dialysis (Cuprophane—Bamberg, Neflex—Union Carbide, or Nephrophane—Filmfabric Wolfen, VEB, Wolfen DDR, East Germany) are made wider, so that twin coils are no longer necessary. The blood flow resistance in some of the coils is still a problem and ultrafiltration is not always what is needed (either too much or too little) for a particular patient. As long as coil kidneys are used, the problems of leaks will be present. Occasionally, leaks have almost prohibited their use and, certainly, they have made coils expensive.

Capillary Kidneys

The Cordis-Dow capillary kidney (11) has been tested in thousands of dialyses (12). The Abcor capillary kidney (13) is being clinically tested, and the Monsanto capillary kidney has capillaries of clot-resisting materials, and is therefore unique, but not yet available. Capillary kidneys have a small filling volume and will undoubtedly gain in popularity.

Price Comparison

The price of disposable coil and capillary kidneys is about the same. The reuse and resterilization of large flat-plate kidneys, small flat-plate kidneys and coils (14), and capillary kidneys has become an economic necessity (15). They should be reused only on the same patient. Resterilization has introduced additional dangers. Moreover, it is often not realized that it is hard to resterilize a kidney and flush it with sterile fluids for less than five dollars, thereby partly defeating the purpose for which the resterilizing was undertaken in the first place. We agree with Nose (16, 17) that the answer, at least for some years, will be an inexpensive disposable insert. We have adapted the artificial kidney developed by Klinkmann's group in Rostock (18), into an American version which takes a disposable insert (15) and is called the "Easy S Kidney." The "S" stands for semidisposable and also indicates the shape in which the cellophane is draped in this type of kidney. The technical development of this kidney was done by David Radford of Vital Assists, Inc. in Salt Lake City, Utah.

Dialyzing Fluid Delivery System

Dialysis fluid without glucose is becoming fashionable, both in Europe (Alwall's group, 19) and in the United States. Apart from a reduction in cost, the absence of glucose suppresses bacterial growth in the dialyzing fluid. The patient is supposed to eat during dialysis to maintain his blood sugar.

Proportioning Systems

The years of fail-safe, fully automatic, beautifully engineered and formica-covered dialyzing supply systems are over. The experience of one of the largest and most successful producers, Milton-Roy (20), coincides with our own (15). Engineers loved them, a few patients, such as a pharmacist and an airline pilot, enjoyed them, but the majority of patients and housewives said that there were too many buttons and too many lights. We are, therefore, going back to simpler systems. Here we have the controversy between proportioning systems and batch systems. If a proportioning system is chosen, a simple one should be considered. About the simplest one seen by this author is the one that Tenckhoff (4) uses to mix his peritoneal lavage fluid. It consists of a roller pump containing a fat and a thin tube. To adjust the volume of the concentrate, he stretches the thin tube. This system may even be surpassed by a system being developed by Klaus Kopp which employs the Venturi principle. Whatever mixing system is used, a conductivity meter is needed to indicate that it is working correctly and at this point, problems arise. Even when a conductivity meter appears to indicate a correct value, it is not necessarily true. It is an unavoidable prediction that, with proportioning systems, patients will continue to succumb from time to time. However, proportioning systems are small, fashionable, and have sales appeal.

BATCH TYPE

Much simpler is the batch type dialysis system, and we have now chosen a 200-liter tank. In this case, an error can be made only once, and hopefully, it will be detected at the beginning of dialysis. The use of tap water, with or without dionizers and water softeners, introduces a set of dangers for the dialysis patient. Copper intoxication, which has led to unexplainable hemolysis and death is one such danger; the hard-water syndrome is another example, not to mention iron, fluoride, chlorides and mercury.

SMALL VOLUME RECIRCULATING DIALYZING FLUID

Greenbaum of the Marquardt Corporation, together with Gordon and Maxwell (21) in Los Angeles, have now brought to a promising degree of perfection a system in which a very small volume, perhaps one liter, of dialyzing fluid is recirculated over an artificial kidney and then brought into contact with urease which breaks down the urea to ammonia, zirconium compounds which absorb the ammonium ion, and charcoal which adsorbs other retention products. Since calcium is inadvertently adsorbed, $CaCl_2$ must be added continuously to the return fluid. This system makes the patient independent of the local tap water supply, the amount of concentrate is minimal, and the ingredients do not seem to be prohibitively expensive. Machines have been clinically tested enough to make for hopefulness for the future, and the writer, at this time, would not invest in any company which concentrates its major efforts on building large volume dialysis delivery systems. Grossman (22) mixes urease with some kind of water-absorbing powder such as ethylene glycol. This not only removes a quantity of plasma water with its electrolytes (which are normally removed by ultrafiltration), but also reduces urea to ammonia, and contains adsorbents to bind the ammonium ion. One might imagine that a coil kidney could be unrolled and that the polyethylene glycol mixture would be powdered on the cellophane coils; the whole thing could be wrapped in a diaper—in this fashion combining modern technology and chemistry with the old and well-known absorbent capacity of diaper material. When the diaper is wet, it has to be changed.

REMOVAL OF LARGER AND SMALLER MOLECULES

What uremic toxin is and what it really does is something which still worries a number of scientists as indicated by a recent review article.[1] For a physician engaged in dialysis treatment such as is the writer, it is wise to assume that uremia is caused by an aggregate of many products that are normally excreted but which are now being retained, and it behooves him to remove them all. It is often surmised that insufficient relief of uremic symptoms is due to the inefficiency of presently used dialysis membranes. It may be true that membranes now in present use are less than ideal but this conclusion cannot be made on the basis of dialyses which take place two or three times per week. The more often one dialyzes, the better one can improve the clinical condition of the patient, and this is particularly true in young children. Urea must be removed, since it is continuously formed in quantities varying from 10 to 40 grams per day. Christopher and Popovich (23) are trying to resolve the relative significance of removal of large and small molecules by varying, for a series of dialyses, the rate of blood flow, dialysate flow, or the surface of the dialyzing membranes, and by studying the effect on neuropathy and other symptoms of uremia. It has been customary to plot dialysance (or clearance) versus blood flow or versus dialysate flow. It will be remembered that the dialysance curve flattens whenever a limiting factor appears. For molecules of medium size the limiting factor becomes the membrane area. Once the dialysance is limited by the membrane area, further increase of blood flow or dialysate flow has no effect. Scribner and Babb and co-workers (24) speak of the "square meter-hour concept." If the main concern is the removal of medium size molecules, and since their removal is determined by the surface area of the membrane, high blood flow or high dialysate flow offers no advantage and it is only the duration of dialysis that counts. We are, however, also concerned with the optimal removal of small molecules. After all, 60–70 percent of the total osmotic load, which is removed during dialysis, consists of substances of molecular weight of less than 100 (urea is 60). Kopp believes that the removal of these smaller molecules should not be neglected and that a measurement of total blood flow through an artificial kidney is a better determination of the effect of dialysis than a record of the dialysis time (see later under dangers). He introduced the kilogram-liter blood flow concept.

OTHER MEMBRANES

Eric Selegny, polymer chemist in Rouen, France, is able to graft enzymes to membranes. So far, he has done this with the enzymes that promote the CO_2 and oxygen transport but not with enzymes that promote the transport of retention products in uremia. The usefulness of such endeavor should be recognized.

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[1] *Arch. Intern. Med.* 126(5): 1970.

Regenerated cellulose is still preeminent in the field. Work with other synthetic membranes is under way but progress has been slow. Membranes used in the Monsanto capillary kidney have already been mentioned. Lyman (25) is constructing synthetic membranes of blocked polyurethane. The aim is to make membranes of sufficient strength, probably hydrophilic, friendly toward thrombocytes, and nonthrombogenic. Andrade (26) has a number of standard tests available by which various membranes can be compared. Labeling with radioisotopes and determination in scintillation counters greatly facilitates this work. Attempts to make new membranes by cutting holes with lasers, or by bombarding materials with neutrons are still experimental. It is to be hoped that within the next ten years, better membranes will be available. Finally, let us not forget that in most artificial kidneys, the resistance to transfer resides not only in the membrane (30 percent for urea), but also in the blood film (40 percent), and in the dialysate compartment (30 percent). Improving the transfer through the membrane by putting in a more permeable membrane does not magically increase the dialysance of a given artificial kidney.

VARIATIONS FROM CONVENTIONAL HEMODIALYSIS

Snyder (27), contrary to what others are doing, removes large amounts of retention products by fast dialysis of plasma which has been separated by centrifugation from the cellular elements of the blood. To become practical this method will require simplification. Extremely interesting is the approach by Henderson and associates (28) in Philadelphia, who use hemo-dia-filtration (29). They are making capillary kidneys of membranes produced by Amicon. These membranes can ultrafilter more than 100 liters during the course of one hemodialysis. It is necessary to dilute the blood before it enters the machine, which otherwise will become obstructed. The use of an enormous amount of ultrafiltrate is a technical problem, which, of course, can be solved if the ultrafiltrate is purified and reused, as would be possible by the Marquardt technique, or if Tenckhoff's peritoneal dialysis machine could be used to prevent the loss of the ultrafiltrate. With this method of hemodiafiltration, very large molecules such as inulin are removed with a clearance which is equal to that of urea. This method, is as yet, not practical, but if it can be combined with adsorption from the dialysate, one may predict a future for it.

OTHER WAYS TO REMOVE RETENTION PRODUCTS

Artificial cells, developed by Chang (30) who has been able to put urease inside the cells, have now been applied in clinical hemodialysis. It is, at this time, not possible to foresee whether conventional dialysis combined with the Marquardt method or the artificial cell technique will ultimately be the treatment of choice. If one were forced to make a guess for the long-range future, it might well be toward the artificial cells. Perhaps it is not even necessary to form whole artificial cells if one wants to employ enzymes. The Corning Glass Company has developed complex shapes of polymers, in the crevasses of which enzymes are so well protected that they cannot be reached by antibodies, and yet can continue to exercise their function. A combination of the enzyme-loaded polymers and charcoal coated with hydrophilic substance, such as Hydron, seems to be extremely promising.

Ever since the work of Yatzides and confirmed by ourselves (31), it has been known that many retention products can be removed by charcoal, if it is protected by a hydrophilic, nonthrombogenic material, of which the polyhydroxy-ethylmethacrylate Hydron is an example. The combination of conventional dialysis, which removes urea rather well, with some modification of the charcoal or resin technique to bind the larger molecules, which conventional dialysis removes poorly (32), is worth considering.

ACCESS TO THE BLOODSTREAM

Arteriovenous shunt cannulas need improvement. Goldberg & Kanter (33) are using cannulas provided with dacron funnels anastomosed to veins previously expanded by A.V. fistulas at a place where the vein is wide because of branching. The Brescia Cimino A.V. fistulas are becoming more and more popular. Unfortunately, to create a good fistula requires the skill of an experienced surgeon. It is essential that the fistula be made with a small vein, as low as possible on the underarm so that a network of veins can develop. Anastomoses of the end of a vein to the side of an artery seems preferable. The danger of large shunts is heart failure, and this is usually not recognized (18). A number of patients may have succumbed to this type of heart failure as a consequence of two fistulas, put in at the same time and both too large, (the second was put in just for safety). A shunt of 500 ml may increase the cardiac output many liters. If there are no vessels available for making a satisfactory Cimino fistula (18), it should be remembered that Wesolowski is able to graft dacron vessels of 4 mm diameter. Saphenous vein grafts have been used. A self-sealing silicone rubber vessel is under development. A company in the Netherlands makes fibrin tubes. The emphasis in the future will probably be on the production of artificial vessels rather than on the use of the patient's own vessels.

SINGLE-NEEDLE DIALYSIS

To insert one needle into a distended vein may or may not be difficult but to insert two needles is far more difficult, so there is great need for single-needle dialysis. Twiss (34) used a single Shaldon cannula in acute dialysis. It should be adapted for use in the Brescia Cimino fistula patients. Kopp has developed an automatic pressure regulated system for single-needle dialysis, using a Brescia Cimino fistula. It has now been applied successfully for many months and we hope that it will soon be available.

DANGERS OF DIALYSIS

Infection from arteriovenous shunt cannula and hepatitis are everpresent dangers. In England, some dialysis units have been closed and others have changed to disposable dialyzers because of the danger of infection to personnel whose task it is to assemble and disassemble kidneys in the dialysis unit. In some areas, a special ward has been established for patients (and personnel) known to be infected with hepatitis. Cellophane membranes which are packaged as disposable envelopes should reduce the occurrence of contamination with blood. A common mode of infection has been the piercing of a tube when a blood sample is taken. The needle goes through the tube into the technician's finger or leg or knee. An impenetrable sleeve will prevent this accident. This sleeve has a slot on the top and can be made from the holders in which disposable syringes are packed. Home dialysis offers the best possible protection against hepatitis Contrary to what is generally believed, death from accidental hemorrhage is rare. A large hemorrhage is easily detected. One death occurred when an indwelling femoral vein cannula became disconnected in a patient during sleep, while he was not being dialyzed. It is said that some cannulas have been cut with suicidal intent. Incorrect composition of dialyzing fluid continues to produce some fatalities, and this will continue to happen as long as proportioning machines are used. The alarms annoy the patient and therefore he decides to cut them out. Not so well known, but very real, is the danger of air embolism. With the advent of the fistula, the use of blood pumps became a necessity, and with the use of pumps, the danger of air embolism is increased. Most bubble detectors, although they will record a large bubble, may fail to record fine bubbles or foam.

Disequilibrium syndrome occurs when the blood urea falls rapidly and the urea content of the brain lags behind. This can certainly be avoided (35) by frequent short-time dialysis and by starting slowly when the patient is first subjected to a series of dialyses. The addition of urea 30 percent of initial blood concentration to the bath can be done if a large dialysis is necessary.

The greatest danger for any patient is when he is under dialysis. When assistants become impatient or less devoted, patients will hardly dialyze the prescribed 6 hours. When the blood flow is small, this may not be enough for adequate dialysis. Kopp prescribes dialysis, not in terms of time, but in terms of the total amount of blood which flows through the kidney, the kilogram-liter blood flow concept. The liters of blood that have to flow through the kidney for adequate dialysis happen to be equivalent to the body weight expressed in kilograms. To eliminate the blood flow, he has introduced a simple revolution counter which can be placed on top of most commercial blood pumps available in the United States. It must be realized that the revolution counter will overestimate the flow if the tube in the pump is collapsed. For accurate measurement of flow, the counter should be combined with a subatmospheric (so-called, negative) pressure detector in the blood line from the patient to the pump. The patient is told to dialyze himself for a certain number of revolutions as estimated by his body weight from a simple table.

HOME DIALYSIS

Home dialysis is ideal. It reduces the cost of treatment with the artificial kidney, but it is geared to the affluent suburban population. Gordon & Cattell (36) suggested a prefabricated room for dialysis, which could be constructed as a mobile unit. One problem will be to convince insurance companies that it is less expensive to have such a unit than to pay for a room in a hospital. For people without help, without homes, and without trailers, satellite centers should be set up. The personnel for staffing these satellite centers can be "dialysis instructors" instead of registered nurses. There is resistance to accepting paramedical personnel for this kind of duty, but it is well known that the shortage of nurses is serious. Well-trained dialysis instructors might well be preferable to nurses in helping persons who are sustained with maintenance dialysis. Unattended overnight dialysis has not fulfilled the high hopes once held for it. It is too complicated and, if the patient sleeps, the spouse does not. More general use of artificial kidneys with higher dialysance makes 6-hour dialysis three times per week or 5-hour dialysis four times per week preferable. This is generally better done in the evening than during the night. Yet, an acceptable, safe, uncomplicated, fully automatic system would help the patient to become more nearly self-sufficient. The aspects of human engineering have been neglected in many of the present machines. They therefore neither convey confidence to the user nor can they be used without mistakes being made. The increasing favor of arteriovenous fistulas which require pumps, and single-needle dialysis which, hopefully, will gain popularity rapidly, make the use of automatic controls mandatory. It is time to re-evaluate equipment for fully automatic and potentially unattended dialysis.

IMPLANTABLE ARTIFICIAL KIDNEYS

Salisbury, many years ago, suggested the use of a dialysis tube coiled up in the peritoneal cavity as an implantable artificial kidney (37). The time has now come when any of the very small capillary or flat plate dialyzers could be used as such. It could be connected with a similar capillary or flat plate dialysis system on the outside of the peritoneal cavity. The two would form a closed system. Cartridges of some kind should be provided, probably containing polyvinyl alcohol to bind water, urease to split the urea, zirconium compounds to bind the ammonium ion, and whatever other resins and charcoal may be needed either to adjust the electrolytes or adsorb retention products. Liquids can, of course, also be removed by ultrafiltration in the outer dialysis system. Conceivably, capsules containing charcoal, etc., could be poked in and out of a compartment in the peritoneal cavity. By the time we will have solved the technical problems of the implantable artificial kidney, our colleagues in transplantation immunology will probably have overcome the problems of rejection, so that the ones to benefit will be those who conscientiously object to transplantation and others in whom transplantation, for some reason, does not work. Ultimately, we shall with confidence offer the patient a choice between dialysis and transplantation.

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RENIN ASSAY IN HYPERTENSIVE DISEASE 7046

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INTRODUCTION

Advances in the understanding of the physiology of the renin-angiotensin system and the development of new assay techniques during the past ten years have led to renewed interest in the role of the renin-angiotensin system in renovascular hypertension and in hypertensive disease associated with renal parenchymal disease and with mineralocorticoid excess. Plasma concentrations of renin and angiotensin are too low to allow direct bioassay. Improved bioassay methods, which measure the product of the reaction of renin on its substrate, and the development of highly sensitive and specific immunoassay methods have made it feasible to examine quantitatively the role of the renin-angiotensin system in the physiologic response to changes in sodium intake or posture and in various pathologic states. Renin assay has been applied widely in the diagnosis and management of various forms of hypertensive disease, but as assay methods differ and patients have been examined under varying conditions of dietary sodium intake, drug therapy, and posture, it is often difficult to compare directly the results of different investigators. Before attempting to review the results of renin assay in hypertensive disease, therefore, a brief outline of the renin-angiotensin system and consideration of methods available for renin assay seems warranted.

Renin, an enzyme of about 40,000 molecular weight (1), is present in the juxtaglomerular cells (2) which, together with the macula densa, constitute the juxtaglomerular apparatus (JGA). While the precise stimulus for renin release is not known (see reviews by Davis, 3, and Vander, 4), several lines of experimental evidence suggest that both changes in intrarenal pressure (5, 6) and intratubular sodium concentration (7) may play a role in the regulation of renin release. Under most circumstances renin is the rate-limiting component in the renin-angiotensin system. Renin cleaves the decapeptide angiotensin I from renin substrate, a circulating $\alpha 2$ globulin. Angiotensin I is converted to the octapeptide angiotensin II by the enzymatic removal of histidyl-leucine; in vivo this reaction occurs during passage through the pulmonary capillary bed (8). Conversion of angiotensin I to angiotensin II has also been found to occur under the influence of plasma-converting enzymes (9). The final product, angiotensin II, is thought to bind to receptor sites in vascular smooth muscle, adrenal cortex, and kidney (10, 11). Angiotensin II appears to be the major mediator of the biologic effects of the renin-angiotensin system.[1] Angiotensin is rapidly degraded by plasma and tissue peptidases, collectively termed angiotensinases (14).

Renin has been assayed both by measurement of the quantity of substrate consumed or of the products formed under controlled conditions of incubation of plasma with endogenous or added substrate. It should be borne in mind that, as neither renin nor renin substrate has been isolated as a pure protein, present assay methods only measure "effective renin activity" which is related not only to the actual quantity of enzyme present but also to the effects of accelerators and inhibitors of the reaction as well as to the action of angiotensinases. The methods of Lever et al (15) and Skinner (16) obviate some of these factors by extraction of renin and inactivation of endogenous substrate but still do not meet rigorous criteria for the measurement of enzyme concentration (17).

Most bioassay methods measure the final product of the reaction (angiotensin II) by its pressor activity in the anesthetized rat rendered more sensitive by ganglionic blockade or vagotomy and, in some instances, prior nephrectomy. The lower limit of sensitivity of most rat bioassay preparations is about 1 nanogram A_{II}. Attempts to measure very low concentrations of renin by prolonging the time for angiotensin generation are rendered difficult by the increasing action of angiotensinases during prolonged incubation. While, in experienced hands, bioassay yields highly reproducible results in renin assay, the method is cumbersome and, moreover, since it is a pressor response which is measured, specificity is lacking.

Immunoassay techniques have been applied to renin assay by measurement of the quantity of angiotensin I generated during incubation of plasma in the presence of inhibitors of plasma converting enzyme and angiotensinases (18–21). These methods are highly reproducible and, with antibodies of high avidity, are considerably more specific and sensitive than bioassay procedures, measuring quantities of angiotensin I as small as 20–50 picograms. Antibodies raised against angiotensin I coupled to protein (21), polylysine (18), or adsorbed to charcoal (19) exhibit little cross-reactivity with angiotensin II. Some antibodies fail to discriminate between intact angiotensin II and its degradation fragments (12); if this should also prove to be the case with antibodies to angiotensin I, it might be a source of error in the application of immunoassay to renin assay.

RENIN ASSAY IN HYPERTENSION ASSOCIATED WITH RENAL ARTERY STENOSIS

Goldblatt's observation that constriction of the renal artery in the dog resulted in elevated blood pressure (22), and the subsequent demonstration that renal artery clamping causes the release of renin into the renal venous blood (6), led many investigators to conclude that the renin-angiotensin system was responsible for hypertension associated with renal artery constriction. With the availability of newer techniques, closer examination of the changes in renin activity and plasma angiotensin II concentration in animals subject to renal artery clamping and in patients with renovascular hypertension have cast some doubt on the role of the renin-angiotensin system in the pathogenesis of hypertension. In the sheep (23) and the dog (24, 25) it has been noted that while plasma renin activity is increased for the first few days following constriction of the renal artery, plasma renin activity usually returns to normal levels during the phase of chronic sustained hypertension. Increased sensitivity to circulating angiotensin does appear to develop during prolonged subpressor infusions of angiotensin II in the rabbit (26) and in man (27), and might be postulated to account for the maintenance of elevated blood pressure despite normal renin activity. The role of angiotensin in the pathogenesis of experimental hypertension has more recently been examined in angiotensin II- immunized animals. McDonald et al (28) and Johnston et al (29) found that the course of renal clip hypertension in the rabbit was unaltered by immunization against angiotensin II. The finding that hypertension developed and was sustained despite biologically effective immunization against angiotensin II strongly suggests that increased activity of the renin-angiotensin system was not responsible for hypertension in this experimental model.

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[1] The heptapeptide has both pressor (12) and steroidogenic actions (13) but the physiologic role of this fragment is not clear.

Although the role of the renin-angiotensin system in the pathogenesis of renovascular hypertension is still in question, measurement of plasma renin activity has contributed to the diagnosis and management of hypertension associated with renal artery stenosis. Renal artery stenosis due to atherosclerosis is not an infrequent angiographic finding in hypertensive patients but, as similar lesions may be found in normotensive individuals undergoing aortography (30), the mere presence of renal artery stenosis does not establish a renovascular etiology of the hypertension. Lacking specific physiologic criteria for the diagnosis of renovascular hypertension, renal artery lesions can be classified as "functionally significant" only if vascular repair or nephrectomy results in sustained lowering of blood pressure. Renin activity in peripheral venous plasma is increased in many patients with radiographic evidence of renal artery stenosis but this measurement fails to identify patients in whom surgical correction alleviates hypertension. While the majority of patients with "functionally significant" renal artery stenosis manifest increased plasma renin activity, surgery fails to relieve the hypertension in almost 30 percent of patients with elevated peripheral vein renin activity (30–35). Failure to alleviate hypertension in patients with elevated peripheral renin activity might be attributed to inadequate vascular repair, to the presence of inapparent arterial disease in the contralateral kidney, or possibly to the effects of arteriolar sclerosis in the "unprotected" kidney (36, 37). It seems equally possible that in these patients elevation of plasma renin activity was due to factors unrelated to arterial stenosis, such as dietary sodium intake or the intrarenal vascular changes of malignant hypertension (46).

Peripheral vein renin activity is normal in 30 to 40 percent of patients in whom surgery proves successful (31, 32, 35). The finding of normal plasma renin activity in patients with proven renovascular hypertension suggests that other factors may influence peripheral renin activity in patients with significant renal artery stenosis. Warren & Ferris (38) found that plasma renin activity in patients with renal artery stenosis could be suppressed to normal levels by salt loading and administration of deoxycorticosterone. Bianchi (32) found increased plasma volume in patients with renovascular hypertension and normal plasma renin, lending further support to the thesis that alterations in sodium balance might affect plasma renin level in patients with renal artery stenosis. Whatever the mechanism by which renin levels are maintained in the normal range, these observations provide further evidence that the elevation of blood pressure is not due solely to increased activity of the renin-angiotensin system.

Judson & Helmer (39) suggested that comparison of renal vein renin concentration from the involved and contralateral kidney might obviate some of the errors attributable to differences in sodium intake, plasma volume or other factors affecting renin release, and improve the prognostic value of renin determination in selecting patients for surgical management. Vascular repair or nephrectomy has been successful in 80 to 100 percent of patients in whom 1.5-fold disparity in renal venous renin concentration has been demonstrated (31, 33, 34, 39–42). Cure of hypertension is less common (25 percent) among patients in whom renal vein renin concentrations are not significantly abnormal (33). It should be recognized that increased renin concentration in renal vein blood does not necessarily indicate increased renin secretion by the affected kidney since renal blood flow is often reduced in kidneys with artery stenosis. Woods & Michelakis measured renal plasma flow in 17 hypertensive patients with disparate renal vein renin concentration and found increased renin secretion by the suspect kidney in only twelve (40).

In summary, measurement of renin activity in peripheral venous plasma appears to be of limited value in the evaluation of patients with renal artery stenosis for corrective surgery; the comparison of renal vein renin concentration appears to provide a somewhat more reliable guide in predicting surgical success or failure. Application of the more specific technique of radioimmunoassay to the assay of renin, and the direct measurement of circulating levels of angiotensin II in patients with renal artery stenosis may improve the predictive value of this form of evaluation.

PLASMA RENIN ACTIVITY IN HYPERTENSION ASSOCIATED WITH RENAL PARENCHYMAL DISEASE

Published reports (43–45) of plasma renin activity in patients with hypertension associated with renal parenchymal disease, generally chronic glomerulonephritis or chronic pyelonephritis, reveal a wider range of values than that seen in normal subjects; if patients with accelerated or malignant hypertension are excluded, no consistent elevation of plasma renin activity is found in patients with renal parenchymal disease. Patients with marked diastolic hypertension and retinopathy (exudates, hemorrhages, and papilledema) almost invariably have marked elevation of plasma renin activity (43, 46) and biochemical evidence of increased aldosterone secretion, which has been attributed to increased activity of the renin-angiotensin system (47). Hollenberg et al (46) suggested that increased renin secretion might be due to marked reduction in cortical blood flow in patients with accelerated hypertension. Brown et al (48) and Stokes et al (49) found that plasma renin activity returned toward normal when blood pressure was effectively treated with hypotensive agents.

Plasma renin values are usually within the normal range in patients with chronic renal failure (50, 51). During maintenance hemodialysis or peritoneal dialysis, plasma renin activity varies widely in response to changes in fluid volumes and sodium balance (51). Blood pressure can be well controlled by frequent dialysis and sodium restriction in the majority of patients with end-stage renal disease. In a small group of patients reported by Vertes et al (50) and Onesti et al (52), plasma renin values were markedly elevated prior to the institution of dialytic treatment and satisfactory control of blood pressure could not be achieved by restriction of dietary sodium intake and removal of extracellular fluid by ultrafiltration. These patients responded to bilateral nephrectomy with fall in plasma renin activity to undetectable levels and return of blood pressure to near normal values, suggesting a possible contribution of the renin-angiotensin system to the maintenance of hypertension in these patients.

PLASMA RENIN ACTIVITY IN PRIMARY ALDOSTERONISM AND OTHER FORMS OF MINERALOCORTICOID HYPERTENSION

Plasma renin activity is suppressed by salt loading in normal subjects (53) and, experimentally, the administration of deoxycorticosterone with salt results in decreased plasma renin activity (38, 54). Marked suppression of plasma renin has been described in most patients with aldosterone-secreting adrenal adenomas (44, 55). Plasma renin is generally unmeasurable or near the lower limits of detectability and, unlike normal subjects, patients with Conn's syndrome fail to demonstrate increase in renin activity in response to sodium restriction, quiet standing, or diuretic administration (44, 55). Prolonged administration of spironolactone in high dosage has been reported to reverse the renin suppression and hypokalemia in these patients (56), though it is not clear whether this effect is mediated through the diuretic action of the drug or correction of potassium depletion.

The measurement of plasma renin activity has been of value in differentiating patients with aldosterone excess due to adrenal adenoma from patients with increased aldosterone secretion associated with severe or malignant hypertension, in whom renin activity is almost invariably markedly increased (55). As a means of detecting patients whose hypertension is due to an aldosterone-secreting adenoma, the finding of low plasma renin activity, when associated with hypokalemia, is suggestive but not conclusive. It is now recognized that as many as one-third of patients with the findings of increased aldosterone secretion, hypokalemia, and suppressed renin activity have not an adrenal adenoma, but diffuse nodular adrenal hyperplasia or normal adrenal architecture (57–61). In these patients, a simple causal relationship between aldosterone excess and hypertension does not appear to exist since total adrenalectomy, while ameliorating the excess aldosterone production and hypokalemia, fails to improve blood pressure. It is difficult to distinguish between aldosteronism due to adrenal adenoma and "idiopathic aldosteronism" associated with adrenal hyperplasia by clinical or biochemical features, though as a group patients with adrenal adenoma exhibit lower plasma renin activity (59), somewhat greater aldosterone production and more profound alterations in serum sodium, potassium, and bicarbonate (61). The stimulus for adrenal hyperplasia and excessive aldosterone secretion is not known. In a small number of patients, hypertension, increased aldosterone secretion, and low plasma renin appear to be due to partial 17-hydroxylase deficiency and excess ACTH as the manifestations are reversed by administration of dexamethasone (62, 63). Hypertension, hypokalemia, and suppressed plasma renin activity have also been described in 17-hydroxylase deficiency (64) where excessive secretion of deoxycorticosterone and corticosterone has been demonstrated, and in patients ingesting large quantities of licorice containing glycyrrhizinic acid (65, 66). Lastly, plasma renin activity is suppressed in the group of hypertensive patients described by Liddle (67), in whom excessive tubular sodium reabsorption and potassium secretion appear to be independent of mineralocorticoid hormone.

Plasma Renin Activity in Essential Hypertension

Suppressed plasma renin activity, unresponsive to salt restriction, quiet standing, and diuretics has been reported in 20 to 40 percent of patients otherwise classified as having essential hypertension (68–76). Conn suggested that such patients have "normokalemic primary aldosteronism" and estimated the incidence of primary aldosteronism to be 20 percent of the hypertensive population (77), but other investigators (68–72, 74–76) have not found increased aldosterone secretion or excretion in patients with essential hypertension and suppressed renin activity. Increased extracellular fluid volume (76) and total exchangeable sodium (75) have been noted in hypertensive patients with suppressed plasma renin activity. While sustained overproduction of aldosterone has not been demonstrated, failure of the adrenal to decrease aldosterone secretion in response to acute dietary salt loading has been demonstrated in some patients with essential hypertension (78) and might account for excess body sodium and suppressed plasma renin. Melby (79) found increased secretion of 18-hydroxydeoxycorticosterone (18-OH DOC) in some hypertensive patients with suppressed renin, suggesting the possibility that this or a related mineralocorticoid might be responsible for altered sodium metabolism and suppressed renin activity in essential hypertension.

Though the finding of suppressed plasma renin activity has led many investigators to search for mineralocorticoid excess in patients with essential hypertension, an alternative possibility might be considered. In the light of experimental evidence that renin release is regulated, at least in part, by a renal baroceptor (5, 6), suppressed renin activity could be attributed to increased intrarenal pressure in patients with essential hypertension (80). Utilizing measurements of wedged renal vein pressure, it has been suggested that increased systemic arterial pressure is transmitted beyond the glomerular arterioles and results in increased peritubular capillary pressure. Intrarenal pressure, through its effect on peritubular fluid dynamics, appears to play a role in the regulation of tubular sodium reabsorption (81). In essential hypertension, there is evidence of excessive proximal sodium reabsorption (82–84); increased intrarenal pressure, by affecting tubular sodium transport, might serve to maintain sodium balance and, at the same time, inhibit the release of renin from the juxtaglomerular cells.

Concluding Remarks

The availability of specific sensitive methods for measuring various components of the renin-angiotensin system has led to a reassessment of the role of renin and angiotensin in the pathogenesis of various forms of experimental and human hypertension. Angiotensin II, acting solely as a pressor hormone, does not appear to account for increased blood pressure in many forms of renal disease associated with hypertension. The recognition that angiotension II exerts an important regulatory control on aldosterone secretion and sodium metabolism (85) suggests that the renin-angiotensin system may still play an important, if less direct, role in the pathogenesis of hypertension. Further study of the inter-relationships of renin release, angiotensin metabolism, sodium balance, and blood pressure control are currently

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BIOGENIC AMINES AND BEHAVIOR[1]

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Introduction

The topic for review is the metabolism of biogenic amines in the central nervous system (CNS) in relation to behavior, from the perspective of neuropharmacology. Historically, the greatest stimulus to the pursuit of relationships between amine metabolism and CNS function was the availability of drugs found to have profound effects on both. The study of amine metabolism has advanced understanding of the actions of many centrally active drugs and it has also been applied to problems of abnormal human behavior. Clearly, the scope of this topic is very broad, and it is necessary to be selective and perhaps arbitrary in limiting the material discussed. Most of the clinical aspects and much of the pharmacology related to amine metabolism have been reviewed intensively and will not be covered (1–13).

The thinking of many neuropharmacologists derives from the assumption that a likely site of interaction between a drug and a neuronal network is the synapse. Their experimentation has usually dealt with the metabolism and function of putative neurotransmitters. For a biologically active small molecule to attain this distinction, it must meet several criteria. The substance and enzymes and precursors necessary for its synthesis must be present in neurons at a presynaptic location. The substance must be released by nerve impulses or a reasonable imitation of physiological stimuli. There must be mechanisms available for inactivation of the released transmitter, and its application to post-synaptic cells should reproduce the permeability changes or functional alterations known to occur during natural transmission. Finally, the effects of drugs on the action of the naturally or experimentally applied putative transmitter should be similar.

There is now evidence that many molecular species may be transmitters or at least "modulators" of neuronal function in the CNS, although nearly all are amines or amino acids (14). They include the well-established peripheral transmitters, acetylcholine and norepinephrine (7, 15, 16), in addition to the aromatic or heterocyclic amines, dopamine (17), serotonin (12), and histamine (18). Other phenolic amines occur in the brain, but their pos-

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sible physiological significance is unknown (19, 20). Newer candidates include excitatory and inhibitory amino acids (21, 22), a small polypeptide (substance P) and the biologically very active fatty acids, the prostaglandins (14). While each of these candidates has fulfilled one or more criteria of neurotransmitters, the catecholamines (CAs), norepinephrine (NE) and dopamine (DA), and, more recently, the indoleamine serotonin (5HT), have had the greatest attention from neuropharmacologists interested in behavior.

BIOCHEMICAL ASPECTS OF AMINE METABOLISM IN THE CNS

The presence of an adrenergic substance in extracts of mammalian brain was discovered in 1946 (23) and the regional distribution of NE in the brain was described in 1953 (24). The highest levels of NE are found in the brainstem and diencephalon. Dopamine, the immediate precursor of NE, is extremely concentrated in the neostriatum, where it may have an independent role in extrapyramidal function (2, 3, 17). In addition, 5HT, a relatively primitive substance, is present in many invertebrate tissues and in the brains of all vertebrates examined. It is most concentrated in the midbrain raphe, hypothalamus, and the basal ganglia as well as the pineal (12).

Biochemical assays of these amines have generally utilized their ability to form fluorescent products, a property further exploited in the development of valuable fluorescence histological methods (25). Neuroanatomists have traced systems of neurons which contain CAs, largely at intensely fluorescent "terminal" varicosities. Such neurons are particularly dense in the hypothalamus, olfactory bulb, retina, limbic system, and certain nuclei of the brainstem, including the locus ceruleus (26). Most of the fluorescence in these locations is due to NE, although DA may predominate in the retina. Extensive evidence has been presented to support a specific role of NE as an inhibitory transmitter at the cerebellar Purkinje cells (16). In the spinal cord, a system of descending axons containing CAs terminates near the cholinergic cells of origin of peripheral preganglionic sympathetic fibers, suggesting that the sympathetic nervous system may have a central adrenergic component. Neurons containing 5HT have also been mapped by fluorescence histology: widely distributed ascending and descending fibers arise from cell bodies of origin in the medial aspects of the brainstem (26). An important conclusion to be derived from such studies is that amine-containing systems are very widespread, and apparently well suited to establishing interrelationships among primitive deeper structures commonly implicated in the central control of visceral and affective functions.

The intracellular location of aromatic amines has been investigated by centrifugal fractionation techniques. A fraction rich in structures which appear to be "pinched-off" nerve endings can be prepared. These "synaptosomes" are enclosed by a plasma membrane which sometimes includes electron microscopically typical synaptic specializations; they contain vesicles, mitochondria, and neural cytoplasm (27, 28). Catecholamines and 5HT have been localized to this fraction, and further localized to the intrasynaptosomal vesicles which may represent a protective storage site.

Enzymes and precursors for amine synthesis occur in the brain. The CAs and 5HT are formed by analogous syntheses, starting from tyrosine or phenylalanine and tryptophan, respectively. The initial reactions are ring hydroxylations which form dihydroxyphenylalanine (dopa) or 5-hydroxytryptophan. These intermediary metabolites are quickly converted to DA or 5HT by a ubiquitous L-aromatic amino acid decarboxylase (29). In certain cells, DA may be β-hydroxylated (30) to form NE. The hydroxylase enzyme activities occur within nerve endings (31–33), although their exact intraneuronal locations are undecided. The initial hydroxylation steps are rate-limiting in the synthesis of the CAs (34) or 5HT (33), although relative rate limitation exists at the β-hydroxylation of DA (35). The regulation of CA synthesis by an inhibitory feedback influence of catechol products upon tyrosine hydroxylase or its pteridine cofactor has been suggested (34), and evidence for it in isolated nerve endings has been presented recently (36). The regulation of 5HT synthesis in the brain is still relatively poorly understood, although availability of tryptophan has been proposed as an important factor (37). A more complete understanding of regulation of levels of amines in the brain is gradually emerging, and the processes of intracellular storage and reaccumulation after use appear to be involved (8).

Mechanisms for inactivation or removal of amines exist in the CNS and are generally similar to those in peripheral sympathetically innervated tissues. Efficient enzymatic mechanisms include deamination by monoamineoxidase (MAO), largely a mitochondrial enzyme, and O-methylation of the CAs by S-adenosylmethionine-catechol-O-methyl-transferase (COMT), the location of which is uncertain (7). The cofactor of COMT is synthesized in the brain (38). The major metabolites of DA and 5HT, respectively, are homovanillic acid (HVA) and 5-hydroxyindoleacetic acid (5HIAA). The major metabolites of NE in the CNS include relatively large quantities of 3-methoxy-4-hydroxy-phenylglycol (MHPG) in addition to vanillylmandelic acid (VMA) and normetanephrine which are produced in much larger quantities in the periphery. Urinary excretion of MHPG has thus been measured in attempts to evaluate indirectly the central metabolism of NE in clinical states (39). The physiological importance of the enzymatic mechanisms of inactivation is unknown. Monoamineoxidase probably acts as a scavenger enzyme to prevent excessive accumulations of amines, and while COMT may act preferentially on released CAs, it is probably not analogous in function to acetylcholinesterase. The process of rapid "re-uptake" at the neuronal membrane is itself probably adequate to account for the removal of CAs after release (8). This process helps to conserve and to regulate intraneuronal levels of amines and it has been under intensive study (8, 40, 41). Transport processes (in and out) at the neuronal surface have been implicated in the actions of many drugs, including the tricyclic antidepressants and the phenothiazines (7), as well as drugs used in treating Parkinsonism (40).

The metabolism of amines in the brain has been studied with radioactive precursors of high specific radioactivity, or the injection of labeled amines directly into the cerebrospinal fluid (CSF), to circumvent the blood-brain barrier to many amines (7). This approach has been particularly useful in studies of NE metabolism, because the uptake and storage of NE are relatively vigorous and specific (8). The usefulness of labeled DA and 5HT is more limited, since their accumulation is much less specific (13, 40, 42). The turnover of amines is estimated by several methods: disappearance of labeled amines (7), disappearance of endogenous amines after inhibition of synthesis (43), and change of specific radioactivity of products after injection of a labeled precursor (44). The CAs disappear in a multiphasic pattern suggestive of rapid synthesis and utilization of some stores and of less metabolic activity of larger reserve stores (7). The overall turnover of 5HT is much faster than that of NE (minutes versus hours), although at least one pool of both CAs may also turn over rapidly. It is probable that 5HT, like other intraneuronally stored substances, exists in multiple pools, although this point is not firmly established. While the estimation of turnover is somewhat more sophisticated than the measurement of static levels, its interpretation is limited since it is a resultant of many variables and because overall turnover may not adequately reflect the function of small active pools.

The disappearance of CAs and 5HT from central neurons has been observed under conditions suggestive of synaptic release. The least physiological approaches to the study of release include prolonged electrical stimulation of various areas of brain to decrease amine levels in vivo (7) and the release of labeled amines from slices of brain in vitro (20). Somewhat more physiological approaches include perfusion through small cannulae in the caudate nucleus to measure spontaneous efflux of DA and its increase by electrical stimulation at the thalamus (45). Various perfusion methods have been used to study the efflux of CAs in the presence of drugs which interfere with their retention (46). Spontaneous efflux of endogenous 5HT into the CSF has been described (47), and stimulation of the midbrain raphe region increased such release at the cortex several-fold, leading to a decrease of 5HT and a simultaneous increase of its deaminated metabolite in the forebrain (48). Furthermore, labeled DA has been released from the retina of the cat by light (49). In general, these approaches to the release of putative transmitters represent imaginative if limited ways of coping with the complicated problem of providing physiological stimuli across specific synapses in the brain. The limitations are that either stimulation conditions are not physiological, or the origin of released material is hard to specify. Probably the light-stimulated cat retina preparation comes closest to being physiological.

The post-synaptic effects of released amines are not well understood. Direct attempts to investigate effects of amines have been made most often by their iontophoretic application onto neurons through micropipettes, while bioelectrical responses were recorded. The results of such experiments are difficult to interpret (50) for they are often contradictory or nonspecific. They suggest that aromatic amines can have widespread effects, either excitatory or inhibitory, in many regions of the CNS (14, 16, 50).

PHYSIOLOGICAL ASPECTS OF AMINE METABOLISM IN THE CNS

Many attempts to define functional roles of aromatic amines have been reported. They do not yet come together into a consistent whole, but their implications are attractive. One approach has been to introduce amines into the CSF or directly into brain tissue. Most often, such injections of CAs or 5HT have led to sedation, sleep, depressed EEG activity and changes in body temperature (13, 51–53). Less often, central excitatory effects of infused CAs have been reported (54). Since it is likely that the biogenic amines have varied and widespread *pharmacological* effects, these findings may not reflect physiological activities. Furthermore, marked differences in behavior result as doses of administered amines are changed (55).

A hopeful trend is the gradual acceptance of quantitative and objective methods of evaluating animal behavior. Thus, for example, drugs which interfere with availability of CAs have led to decreases in locomotion, exploratory behavior, and conditioned avoidance in laboratory animals (56–58). Another model of behavior which has attracted much interest is self-stimulation through electrodes implanted in the lateral hypothalamus, as described by Olds & Milner (59). The appeal of this model includes its similarities to spontaneous appetitive or rewarded behaviors. It usually involves stimulation of the median forebrain bundle, which appears to be a major anatomical pathway by which amine-containing fibers ascend to the forebrain. Such behavior is depressed by drugs which decrease the availability of CAs to synapses, increased by amphetamine (58, 60), but less affected by *p*-chlorophenylalanine, an inhibitor of tryptophan hydroxylation (60), implying that a functional "catecholaminergic" system is probably necessary for self-stimulation. Other studies have led to the suggestion that 5HT may be involved in behavior-inhibiting systems. For example, *p*-chlorophenylalanine can permit increased rates of responding in rats caught in a conflicted approach-avoidance situation (61), and diminish their ability to habituate to novel (62) or startling stimuli (63). The concept that 5HT and the CAs might be involved in contrasting functional systems was also formulated by Brodie (64) in the 1950's, in an imaginative hypothesis in which NE subserved an "ergotropic" or arousal-sympathetic system, and 5HT subserved a "trophotropic" or quieting-parasympathetic system of the diencephalon as proposed by Hess (65). Such speculations, while still premature, are tempting since they bring order to complex experimental observations. One important reservation to be held is that most, if not all, of the drugs used in such experiments do not have simple actions. For example, reserpine and chlorpromazine alter many chemical systems and tend to depress behavior generally, and inhibitors of synthesis, even *p*-chlorophenylalanine, are not absolutely specific for one amine (12).

States of consciousness have also been associated with amine metabolism. Jouvet and his associates have made a comprehensive attempt to relate amine metabolism to sleep and wakefulness (52). Lesions of the raphe nuclei of the brainstem decrease 5HT levels of the forebrain and have produced generalized insomnia in several species (52); stimulation here can induce sedation and changes in the EEG associated with sleep (66). Lesions at the locus ceruleus of the brainstem decrease NE levels rostrally and selectively inhibit paradoxical or rapid-eye-movement ("REM") sleep (52). A variety of pharmacological experiments are also generally consistent with the conclusion that interference with the synthesis of 5HT can inhibit sleep while enhancement of its availability, for example, by giving 5-hydroxytryptophan, can increase the occurrence of sleep, and especially slow wave sleep. Interference with availability of NE tends to diminish "REM" sleep selectively. Other pharmacological experiments are less clearly consistent with this hypothesis (52). Furthermore, the lack of specificity of the brainstem lesions and the fact that behavioral effects of *p*-chlorophenylalanine upon sleep rapidly disappear while 5HT levels remain low have been criticized (67).

Animals have been exposed to many unpleasant situations including exercise to exhaustion, extreme temperature changes, electrical shocks and seizures, hypoxia, overcrowding, or intoxication with drugs including amphetamines and hallucinogens (13, 68–73). The earliest of such "stress" experiments generally noted small decreases of levels of NE in the brain and not much change in 5HT (13). In a somewhat more subtle experiment, NE was more depleted in brains of rats unable to avoid foot shocks than in those able to avoid the shocks by their responses (74). More recently, as the dynamic aspects of amine metabolism have received more attention, acute "stress" has been found to increase rates of "turnover" of amines, presumably reflecting increased synthesis and utilization. More extended exposure to foot shocks has resulted in smaller decreases of NE levels, presumably by some form of "adaptation" (75). One explanation of this observation is offered by findings that tyrosine hydroxylase activity in the brain may increase during chronic stressful stimulation (76, 77). An even more provocative observation is that stress of pregnant rats resulted in a sustained increase in turnover of NE in the brains of their offspring (78). While these findings are all interesting, it is difficult to interpret the results of such crude forms of physical abuse, much less to relate them to human psychological stress. Possibly, conflict situations in animals are a step closer to the human experience. Indirect evidence that psychological stress in man might result in increased discharge of NE in the brain are the findings that stressful situations led to increased urinary excretion of MHPG (79, 80). Whether these findings imply that important physiological relationships exist between amine metabolism in the brain and adaptation to stress is unclear.

Other studies which are in many ways similar to the "stress" experiments, suggest that there may be a relationship between defensive or aggressive behavior and amine metabolism. For example, displays of "sham rage" can be provoked in the cat by electrical stimulation of the amygdala or by transection of the brainstem (81, 82). They result in decreases of neuronal CAs in the brainstem and are enhanced or inhibited by drugs believed to act by enhancing or inhibiting the action of CAs at synapses. Furthermore, aggressive behavior in male mice exposed to overcrowded conditions appears to be either increased or diminished by pharmacological interventions which may increase or decrease the availability of amines in the brain (83). The latter effects are somewhat complicated and appear to involve both CAs and 5HT. The "muricidal" (mouse-killing) behavior of certain rats has also been correlated with decreased levels of 5HT in the brain (83, 84). Whether such experiments reveal metabolic correlates of specific behavioral states or nonspecific increases in neuronal activity is unknown.

One of the striking features of many manifestations of defensive and aggressive behavior is that while they are complex, they are often stereotyped. There are other regularly reproduceable behaviors which can be induced in animals by drugs that alter amine metabolism. Amphetamines induce a bizarre pattern of posturing, sniffing, licking and compulsive gnawing in rats (85) or in cats (86). These effects can be blocked by drugs which inhibit the synthesis of DA and can be reproduced selectively by the application of DA directly to the caudate nucleus (87). It is hard to avoid the conclusion that such actions are probably mediated by effects on the ascending fibers to the neostriatum which contain DA and which have also been implicated in Parkinsonism (2, 3, 17). While these phenomena are interesting, it is improbable that they have direct bearing on the psychological changes produced by the amphetamines, or on the behavior of catatonic schizophrenics, as has at times been suggested.

Another important and rapidly evolving hypothesis is that amines, and particularly CAs, may modify the activity of hypothalamic neurons which secrete pituitary trophic hormone-releasing factors (88). For example, intraventricular injections of DA have led to increased secretion of the releasing factors for luteinizing hormone (LH) and growth hormone (GH) (88, 89). Drugs which deplete CA stores or which may block their actions have been found to inhibit the release of LH and GH (88, 90). Similarly, the secretion of antidiuretic hormone (ADH) has been related to the action of NE (91). Recent findings that L-dopa may provoke increased release of GH in man (92) and that chlorpromazine may inhibit this release have suggested exciting therapeutic possibilities (93). Amines in the brain may also alter the secretion of ACTH: the possibility of a reciprocal relationship between NE and the secretion of ACTH was raised as early as 1962 (94), and the topic has been reviewed recently (95). A further suggestion is that the cyclic diurnal alterations in secretion of ACTH may be related to reciprocal changes in the availability of 5HT in the brain (96).

Several studies also suggest relationships between amine metabolism and endocrinological and behavioral aspects of sexuality. A frequent observation is that males of several species develop compulsive sexuality following *p*-chlorophenylalanine (97, 98). They mount other males, and often become aggressive, even vicious, and appear to be confused (98). There have also been attempts made to relate cyclic changes in the metabolism of 5HT or the CAs to the estrus cycle of female rodents (99–101), which may generally metabolize 5HT faster than males (62). The findings in this field are provocative but as yet unsettled, and at times conflicting (102).

Important relationships have also been proposed for the control of eating behavior. Injections of minute quantities of NE into the hypothalamus of a sated rat can induce eating (103). This effect was potentiated by an inhibitor of reuptake of amines and inhibited selectively by drugs known to block α-adrenergic receptors. Similar effects followed injections of NE into the lateral ventricle of rats made anorexic by a lesion of the lateral hypothalamus, and have been related to a "reward" function of the NE-containing neurons in the median forebrain bundle (104). Inhibition of tyrosine hydroxylase led to decreased bar pressing by rats for a food reward, while other activities were not affected (105), further supporting the analogy between appetitive or rewarded behavior and self-stimulation at the median forebrain bundle proposed by Stein and Wise (58, 104). The complexities of the hypothalamic control of food intake and the nonidentity of food intake control centers and self-stimulation centers of the hypothalamus have recently been pointed out (106), suggesting that a single mechanism to explain all appetitive behaviors mediated by the hypothalamus is premature and unwarranted.

Finally, amine metabolism has been implicated in other homeostatic and autonomic functions of the diencephalon and brainstem, including temperature regulation (53, 107—109). The control of cardiovascular and respiratory functions may also be involved (110—113), and an intriguing recent report noted reciprocal changes of brainstem and cardiac levels of NE in rats instrumentally trained to alter their heart rate, with stimulation of the median forebrain bundle as the reinforcing "reward" (114).

CONCLUSIONS

In summary, it seems probable that neuronal systems utilizing aromatic amines as neurotransmitters are vitally involved in many diencephalic and brainstem mechanisms underlying the autonomic control of fundamental homeostatic mechanisms. Since neurons containing CAs or 5HT represent only a small proportion of the total population of cells in the CNS, it is equally probable that systems utilizing other transmitters remain to be defined by methods modelled after those discussed here. A more speculative possibility is that neuronal systems mediating overall states of consciousness or more complex affective, sexual, or aggressive behavior may also utilize aromatic amines as neurotransmitters.

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ADOPTED CHILDREN AND THEIR FAMILIES IN THE EVALUATION OF NATURE-NURTURE INTERACTIONS IN THE SCHIZOPHRENIC DISORDERS

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The question of the causes of the schizophrenic disorders, which are among, if not the most common of, the serious psychiatric illnesses, has long been a controversial issue in psychiatry. In the past few years, data have been gathered which incontrovertibly demonstrate the role of genetics in the etiology of the schizophrenic syndromes. It is the purpose of this review to summarize these data and discuss the relevance of these, as yet not widely known, studies. Before proceeding, it will be useful to review briefly the definition of the disorders (nosology), the prevalence of the disorders, and the data which have been traditionally employed to support hypotheses of biological or psychological etiologies.

Let us begin with the problem of nosology. The term "schizophrenia" has been variously used to refer to a wide variety of clinical manifestations so that a brief summary of its past, present, and geographic usage may be helpful. In 1896 Kraeplin (1) proposed that certain seemingly disparate psychoses (hebephrenia, catatonia, and certain paranoid states) be separated from the other psychoses and designated "dementia praecox." The illness was characterized by early onset, progressive course, and flagrant psychiatric symptomatology. Bleuler, in 1911, (2) further elaborated on Kraeplin's subgrouping, vastly broadening the category to include less severely ill individuals and renaming the syndrome as the group of "schizophrenias." Bleuler asserted that all the affected individuals had, despite their seeming behavioral heterogeneity, certain "primary" (basic) psychological abnormalities which they held in common. These included cognitive abnormalaties not related to intelligence and manifested as "thought disorder"; abnormalities of emotional reactivity, which included unresponsiveness, marked ambivalence, and alterations in relations with other people; and finally, a predeliction for withdrawal into fantasy. These were the defining, the criterial, attributes of the syndrome. This group of individuals was, Bleuler felt, also subject to more obvious blatant "secondary" (i.e., not necessarily present or essential for the diagnosis) psychopathological manifestations including aberrant motor behavior (catatonia), abnormal perceptual phenomena (illusions and hallucinations), and elaborate intellectual maladaptations (delusions). Bleuler's definition was far more encompassing than Kraeplin's: Bleuler used the term "schizophrenia" to refer to all individuals with the "primary" symptoms, while Kraeplin's "dementia praecox" referred to those individuals who manifested both the primary and secondary symptomatology of Bleuler. (Parenthetically, Bleuler straddled the fence, etiologically referring to the affected individuals as constituting a "group," leaving open the possibility that "schizophrenia" had several distinct subgroups and/or etiologies). I have digressed to explain the historical origin of the diverse manner in which the term "schizophrenia" is construed. In European psychiatry, it is generally used in the narrow sense to refer to Kraeplin's "dementia praecox"; in the United States, the term is applied in Bleuler's sense and includes a much larger spectrum of illness. To digress further, since the question of etiology concerns *what* is to be explained, it would be useful to state that there are three major forms of psychiatric illness designated as "schizophrenic" in current American psychiatry. The first corresponds to Kraeplin's dementia praecox and is variously termed "process," "chronic," or "true" schizophrenia. Individuals with this illness are characterized by a history of poor prepsychotic adjustment, having tended to have been an introverted and "shut-in," having few peer contacts and few heterosexual relationships; they have frequently remained unmarried and have shown poor occupational adjustment. The onset of their illness tends to be gradual and without clear-cut psychological precipitants. The illness is characterized by conspicuous manifestations of the "primary" Bleulerian characteristics; the "secondary" characteristics (hallucinations, delusions, etc.) are often present as well. This illness does not tend to respond well to treatment and its course tends to be chronic.

A second form of psychiatric disorder, designated "reactive schizophrenia" or "acute schizophrenic reaction" in the United States, presents an appreciably different clinical picture. Individuals suffering from this disorder in general manifest a good premorbid personality adjustment in terms of their interpersonal relations, heterosexual adjustment, and work history. The illness tends to have a relatively rapid onset and often appears to have psychological precipitants. The presenting picture is characterized by the clear-cut presence of secondary symptoms and comparatively lesser evidence of primary ones, together with the presence of affect (so that the illness may be considered to be manic or depressive) and a cloudy rather than a clear sensorium. Acute schizophrenic reactions tend to remit in a relatively short period of time and the long-term prognosis is fairly good. The illness itself tends to respond to phenothiazines and electro-shock therapy. European psychiatrists do not consider acute schizophrenia reactions to be related to the schizophrenias as indicated by their designations of it as "schizophreniform psychosis" or "psychogenic psychosis."

A third psychiatric disorder which is considered related to the schizophrenias in the United States, is one designated by a variety of terms, including "pseudoneurotic schizophrenia," "borderline schizophrenia," and "ambulatory schizophrenia" (to present only a few of a host of synonyms). This disorder is harder to characterize symptomatologically (because of the varying proposed diagnostic criteria) but would appear to include individuals having a chronic history of psychological maladaptation with abnormalities in the following areas: 1. thinking—strange, vague, illogical mentation, which tends to ignore reality, logic, and experience and results in poor adaptation to life experiences; 2. affective life—"anhedonia," the inability to experience intense pleasure so that the individual will report a history of never having been happy (although he may never have been seriously depressed); 3. interpersonal relations—a tendency to polar opposites which may include either the absence of deep intense involvement with other people or excessively "deep" and dependent involvement with others; chronic difficulty in sexual adjustment characterized by either a very low sexual drive or a promiscuous chaotic social adjustment which may include a fluctuating mixture of heter- and homosexuality; 4. psychopathology—which is characterized not only by its intensity but by its lack of constancy; most "neurotics" tend to maintain a fixed pattern of maladaptation and remain compulsive, phobic, or hysteric while borderline schizophrenia individuals may show multiple neurotic manifestations which shift frequently (obsessive concerns, phobias, conversion symptoms, psychosomatic symptoms, etc.); severe widespread anxiety and occasionally short-lived episodes which have been designated as "micropsychotic" during which the individual experiences transient delusions, hallucinations, feelings of depersonalization or derealization. The course of the disturbance tends to be lifelong, generally without deterioration, and the illness itself seems fairly refractory to drugs generally employed in the treatment of psychosis. The "pseudo-neurotic schizophrenias" have not, in general, like the reactive schizophrenias, been considered by most European and many American psychiatrists to be part of the schizophrenic syndrome.

The incidence of "true," "process" schizophrenia has been assessed in a number of populations and the median risk of morbidity is about 0.8 percent (Rosenthal, 3). Population morbidity figures are not available for the borderline schizophrenics, but in one study (Rosenthal et al, 4), the ratio of borderline schizophrenics or "near" borderline schizophrenics to "process" cases was nearly 8 to 1 which would suggest that the risk for the entire population would be 6 or 7 percent. This figure must, because of the special nature of the population investigated be taken with caution; although it may seem outstandingly high, its order of magnitude is in keeping with the proportion of (unspecified) seriously distrubed persons found in psychiatric epidemiological studies (e.g., Srole et al, 5). The diagnostic category of borderline schizophrenia is not given credence to nor employed by many clinics; of those that do the diagnosis is common, with one clinic (Gaw et al, 6) reporting that one-half of their applicants are so diagnosed.

In considering the etiology of the schizophrenias what may be at stake then is the etiology of not only a severe but uncommon disorder, "process schizophrenia," but also the etiology of an illness possessed by a large fraction of the psychiatrically disturbed population. The theories concerning the genesis of the schizophrenias have fallen into two major groups which, when stated in their most extreme forms, are that: (*a*) the schizophrenias are genetic abnormalities; (*b*) the schizophrenias are learned patterns of maladaptation produced by exposure to a disturbed psychological environment. Both views are based upon an observation of considerable antiquity, that is, that there is a familial clustering of psychiatric disease. Researchers with the two points of view have traditionally employed two major techniques of investigation.

The consanguinity studies and family studies.—These studies have investigated the frequency of mental disorder in the relatives of schizophrenic patients. These studies have shown that there is a significantly higher prevalence of schizophrenia among the relatives of schizophrenics than there is among the general population and that the closer the biological relationship the greater is the prevalence of schizophrenia in these relatives. This tendency peaks in the high concordance rate of schizophrenia which is seen in monozygotic twins. The results of such studies have been interpreted by the "genetic school" as demonstrating the operation of genetic factors. These studies have been criticized by nongeneticists on a number of grounds. First, the mechanism of genetic transmission is unclear and does not fit any simple Mendelian model. Second, the studies on monozygotic twins have shown that an appreciable fraction of pairs (of the order of magnitude of 50 percent) are not fully concordant for this disorder. Since monozygotic twins ostensibly share an identical genetic complement, the occurrence of discordance must—state the environmentalists—be explained by other than genetic factors.[1] Third, and perhaps more important, it is tenable that the increased prevalence in close family members is a manifestation of intrafamilial learning. Those workers espousing the psychological and environmental theory of etiology would assert that schizophrenia is learned and that speaking schizophrenically, like speaking Swahili, tends to run in families.

The psychodynamic and family studies.—These studies have explored the psychological experiences which schizophrenic patients have undergone and have revealed that schizophrenics have frequently been reared in, and exposed to, a disturbed psychological environment. Such studies have been interpreted to show that the disorder is a learned form of adaptation (which appears as a maladaptation) to the patient's deviant experiences. These studies, too, are subject to a number of critiques. First, many have not employed control groups or have not addressed themselves to the statistical problems which emerge when one considers the prevalence of such disorders in the families of control groups.[2] Second, these studies can be interpreted (and have been by those of a genetic bent) to document the fact that there is an increased manifestation of *form frustes* of schizophrenia among the relatives of schizophrenics. If the genetic theorist is willing to allow that this disorder may occur on a continuum these data would only provide further substantiation of a genetic hypothesis.

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[1] These studies clearly document that genetic factors may not be a sufficient condition for the development of schizophrenia. They do not document that psychological factors necessarily play an etiological role. What is nongenetic may be biological as well as psychological, and biological nongenetic factors have been implicated in one such study (Stabenau & Pollin, *7*) as related to the development of the schizophrenia. It is of some analogical interest that the concordance rate of schizophrenia in monozygotic twins is approximately that of diabetes (Harvald & Hauge, 8).

What makes the interpretation of both types of studies a greater source of heat than light is the inextricable confounding of biological and psychological relatedness. The deviant psychological experiences have usually been received from persons to whom the patient is biologically related. It is impossible to decide whether, or to what extent, this disorder has been biologically or organically transmitted since the abnormalities in the parent may be either a manifestation of the disorder in the parent or a cause of the disorder in the child. The data are compatible with either the biological or the social transmission of schizophrenia and do not permit an evaluation of the relative contributions of either.

In the past ten years a technique for circumventing this problem occurred independently to several people: Heston (11), Karlsson (12), Kety (13), Rosenthal (14), and Wender (15). This technique utilizes the strategy of studying individuals adopted in infancy. Since, in such instances, the parents who provide the genes are different from the parents who provide the psychological environment, the relationship of pathology in either group to the pathology of the offspring can be independently assessed. Interestingly, this method had previously been used thirty years ago to study the roles of nature and nurture in intelligence (Skodak & Skeels, 16), and twenty years ago to study the role of genetic factors in alcoholism (Roe & Mittleman, 17). Why the technique was not applied earlier to the study of psychosis is an interesting question; one major deterrent may have been the significant number of practical problems which had to be overcome to implement these studies.

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[2] This last point requires some further explication. Since schizophrenia is a relatively uncommon disorder and since the degree of psychopathology found in the families of schizophrenics is found in an appreciable number of controls, a simple arithmetic calculation will document that only a very small fraction of persons exposed to such deviant familial experience develop schizophrenia. Logically, it is therefore necessary to postulate the existence of other factors. For example, consider a population of ten thousand individuals, 1 percent of whom are schizophrenic and in which 70 percent of the schizophrenics and 10 percent of the controls have been exposed to deviant familial experiences. (These figures are approximately those reported by Alanen, 9). Obviously, the difference in prevalence of psychopathology between the parents of the schizophrenics and those of the controls is "statistically significant." Looked at another way, the population contains 100 schizophrenics, 70 of whom have had deviant parents, and 9900 nonschizophrenics, 10 percent or 990, of whom have had deviant parents; of 1050 individuals with deviant parenting less than 7 percent have become schizophrenics. Obviously, deviant parenting is at most a necessary condition for producing schizophrenia; it is obviously not sufficient in itself. This statistical pitfall is one into which not only psychiatrists may stumble. (For further elaboration, see Wender, 10).

THE ADOPTIVE STUDIES OF SCHIZOPHRENIA[3]

Employing the general strategy of using adoption to separate the effects of nature and nurture, a number of studies have been conducted in an attempt to provide answers to the following four questions. 1. Do genetic factors play any role in the etiology of schizophrenias, and if so what are the psychological manifestations of the genetic diathesis? 2. If genetic factors are a contributory cause to some schizophrenias, in what proportion of schizophrenias do they play a role? 3. Is deviant raising a necessary condition for the appearance of schizophrenic symptomatology? 4. Does deviant raising contribute to or aggravate the manifestations of the genetic diathesis?

With regard to the first question, whether genetic factors play any role in the etiology of the schizophrenias, there are two relevant studies, those of Heston (11) and Rosenthal et al (4). Both employed the same strategy, that of evaluating the adult personalities of individuals born to a psychotic parent and reared in adoptive or foster homes (Heston) or adoptive homes (Rosenthal). Heston's sample was composed of offspring born to actively (process) schizophrenic mothers and removed from those mothers within the first weeks of life; the children were placed in foundling homes for an average of two years before receiving final family placement. In the Rosenthal study either the children's mother or father was schizophrenic (not only "process" but also "reactive" or "borderline"); the majority of parents were not known to be psychotic at the time of birth [the first hospitalization occurred on the average 10 years after the birth of the child], and the child was placed in an adoptive home within the first two years of life (mean 6 months). In both studies, the schizophrenic's offspring constituted the "index group" and were compared to a "control group" of foster and/or adoptive children whose biological parents were not known to be psychotic and who were matched for age, sex, time, and type of placement. All the subjects were evaluated with a structured psychiatric interview and psychological tests. In the Heston

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[3] The brevity of this review precludes a detailed consideration of the research methodology employed in the studies to be discussed. The interested reader is urged to consult the original articles.

TABLE 1. Schizophrenic Psychopathology in the Reared-Away Offspring of Schizophrenic Parents

<table>
<tr><th></th><th>Process schizo-phrenia</th><th>Borderline</th><th>Schizoid or doubtful borderline</th><th>Other</th><th>Total</th></tr>
<tr><td colspan="6">Heston (1966)</td></tr>
<tr><td>Index cases</td><td>5</td><td colspan="2">8</td><td>34</td><td>47</td></tr>
<tr><td>Control cases</td><td>0</td><td colspan="2">0</td><td>50</td><td>50</td></tr>
<tr><td colspan="6">Schizophrenic+borderline schizophrenics+schizoid vs "Others," p<.001 (exact probability test).</td></tr>
<tr><td colspan="6">Rosenthal et al (1971)</td></tr>
<tr><td>Index cases[+]</td><td>3</td><td>9</td><td>10</td><td>47</td><td>69</td></tr>
<tr><td>Control cases</td><td>0</td><td>5</td><td>7</td><td>55</td><td>67</td></tr>
<tr><td colspan="6">Schizophrenic and borderline schizophrenic vs. "Others," p<.03 (exact probability test).</td></tr>
<tr><td colspan="6">Combined Data</td></tr>
<tr><td>Index cases</td><td>8 (7%)[a]</td><td colspan="2">27 (23%)[b]</td><td>81</td><td>116</td></tr>
<tr><td>Control cases</td><td>0 (0%)</td><td colspan="2">12 (10%)</td><td>105</td><td>117</td></tr>
</table>

The probability that the excess of:

[a] Schizophrenics in the index group is chance, p<.003 (exact probability test).

[b] Borderline schizophrenics or schizoids in the index group is chance, p<.004 (exact probability test).

[+] Offspring of "process" or "borderline" schizophrenic parents.

study, the interviewer was aware of the psychiatric status of the biological parent whereas in the Rosenthal study the interviewer was blind in this regard. Table 1 presents the findings of each study and summarizes the results of both studies taken together.

In summary, we find that about 7 percent of the offspring of process or borderline schizophrenic parents manifest process schizophrenic psychopathology when reared in foster or adoptive homes. This rate is well within the range (7–17 percent) found among the offspring of schizophrenics reared by their parents (Rosenthal, 4). Likewise, 23 percent of the index group develop pathology in the schizophrenic "spectrum" as compared to 10 percent in the control group. This difference is highly significant statistically, (p<.004, exact probability test).

The inference to be drawn from these studies is obvious. Since there is an increased prevalence of schizophrenic disorder among the nonparent-reared offspring of schizophrenic biological parents, there is at least one form of schizophrenia with genetic determinants. Note that I state "one form"—logically, this design can demonstrate only that some schizophrenic parents transmit this disorder to their offspring. This design obviously cannot demonstrate the converse: that all schizophrenias have a genetic component to their illness.

One other finding, not stated in the tables, is of some interest. One question posed was whether the putative schizophrenic genetic load had other psychiatric, but nonschizophrenic, manifestations. Heston's study would suggest that this may be so. Among the 42 index subjects who were not schizophrenic, 13 manifested personality disorders (emotionally unstable personalities and mixed psychoneurotic reactions) versus 7 personality disorders among the controls. The difference is again significant ($p<.05$, exact probability test). Data for the second group of studies also suggest that genetic factors may play a role in the development of other psychiatric disorders. The Rosenthal data have not yet been completely analyzed and as yet it is not known if they will substantiate these findings.

There are a number of additional points that need to be made about interpretation of these studies. The first relates to psychopathology, including schizophrenia, among the controls. In the Heston study, none of the controls fell in the "schizophrenic spectrum ' (but nine were impaired). In the Rosenthal study 10 percent of the control group manifested "schizophrenic spectrum" pathology. This does not necessarily demonstrate that such psychopathology can arise on a nongenetic basis. In neither study were the control parents examined and found to be psychologically healthy; all that is known is that they had never received a psychiatric diagnosis. The probability is appreciable that some biological parents of controls were in the "schizophrenic spectrum" and gene carriers but had never been "officially diagnosed". In the Rosenthal study it was found that of 36 individuals who were schizophrenic, borderline, or schizoid, only one had received psychiatric care (and was thus officially diagnosed). This finding is consonant with that of other population surveys, e.g., that of Srole et al (5) which revealed that only a small fraction of seriously disturbed individuals received psychiatric care. The data available do suggest that the parents of the controls are socially deviant. In addition to the obvious fact of having given up children for adoption, many had histories suggestive of disorganization, including multiple liasions and illegitimate pregnancies. Obviously, a direct test is needed. One technique to circumvent this difficulty would be to interview the parents of the controls and eliminate those whose parents manifest psychiatric disorders; this is currently being done but the results are not yet available.

A second point (which might constitute a logical objection) in these studies is that in the case of the offspring born to actively schizophrenic mothers there might be a form of biological but nongenetic transmission. That is, it is conceivable that biological factors produced by or associated with illness in the mother (e.g., hormonal factors associated with stress, possible abnormal metabolites produced by schizophrenics, inadequate diet, etc.) might influence the development of the fetus and result in psychiatric illness in later life. This variable may be controlled in two ways: (*a*) examining the offspring of mothers who became schizophrenic before and after the birth of the child; (*b*) comparing the offspring of schizophrenic mothers with those of schizophrenic fathers—since the offspring of the schizophrenic fathers receive only the father's genetic load without exposure to a possibly noxious prenatal environment.

A third and related question pertains to the role of environmental factors in the development of the illness. It is, as discussed above, possible that disturbance in both the index and control groups is purely genetic in origin. It might be argued, however, that the psychological and social conditions associated with adoption are psychologically noxious and interact with a biological abnormality to produce schizophrenic illness in the index group. Although this argument cannot be refuted, it still presupposes a biological predisposition, since the control group did not, in general, become ill when subjected to the adoptive procedure. What might be asserted is that genetic factors do exist (are necessary) but are not sufficient.

At this point it is worth noting that virtually no disease is entirely "genetic." If the development of phenylketonuria (generally considered to be a "genetic disorder") could be totally prevented by a phenylalanine-deficient diet and if, in addition, most edible foodstuff did not contain phenylalanine, the development of PKU would be dependent on diet. Since most natural diets include phenylalanine, the disorder is deemed "genetic" rather than "environmental." Lest this example seem far-fetched, consider the example of favism. In countries in which fava beans are a dietary staple, this form of hemolytic anemia is a "genetic disorder," whereas in countries in which fava beans are rarely consumed, the disorder can be regarded as a form of food allergy. It may be the case that a "typical" upbringing is for the child with a schizophrenia genotype, analogous to a phenylalanine-rich diet for the child with PKU. One can extrapolate only to similar environments; all that may be said is that given an apparently adequate psychological environment a genetically predisposed child experiences approximately the same risk of developing a schizophrenic illness as when raised in an apparently deviant environment (i.e., being raised by his schizophrenic parents). It is possible that unusual environments (which were not sampled, e.g., germ-free, dysprosium-rich, sensory-deprived) might prevent the appearance of schizophrenic illness.

A fourth, and cautionary, point relates to social adequacy. These studies relate to psychiatric diagnosis and not to social functioning. As the social adjustment of retardates can be affected by education and rehabilitation, so the social functioning of the adopted offspring of schizophrenics may have been helped by their comparatively normal upbringing. This point is of obvious practical importance.

The first design has answered the question of whether genetic factors play any role in the etiology of the schizophrenias. The second design attempts to answer another question: in what fraction of schizophrenias do genetic factors play a role? In these studies, one begins by examining groups of adult schizophrenics and a comparison group of normals, all of whom had been adopted in infancy, and determining the prevalence of schizophrenic illness in their biological and adopting families. If schizophrenia is a genetically transmitted disorder one should find an increased prevalence in the biological relatives of the adopted schizophrenics, while if the disorder is psychologically transmitted one should find an increased incidence in the adoptive relatives of the adopted schizophrenics. (One would expect to find an increased incidence among the adoptive parents since they had produced the disorder and among the adopted sibs since they had been subjected to the same environment).

Such a study has been conducted and is reported by Kety et al (18). In this study he examined the prevalence of psychopathology in the biological and adoptive families of 33 adult schizophrenics who had been adopted in infancy (the Index group) and in 33 adult adoptees who were not known to have had psychiatric illness and who had been matched with the index group on the basis of age, sex, age of adoption, and social status of the adoptive families (the Control group). The prevalence of schizophrenia and other psychiatric disorders among the biological and adoptive relatives was determined by finding which relatives had been seen at psychiatric clinics and hospitals. (This technique produces a bias which will be discussed below.) Blind raters assessed these records and diagnosed the relatives. The results are shown in Table 2.

TABLE 2. DISTRIBUTION OF SCHIZOPHRENIC PSYCHOPATHOLOGY IN THE RELATIVES OF SCHIZOPHRENIC ADOPTEES AND THEIR CONTROLS

	Relatives with schizophrenia or probable schizophrenia	
	Biological relatives	Adoptive relatives
Index cases (n=33)	11 out of 150	1 out of 74
Control cases (n=33)	3 out of 156	3 out of 83
Significance (exact probability test)	p<.07	n.s.

TABLE 3. Distribution of Psychopathology in the Relatives of Schizophrenic Index Cases and Their Controls

	Relative with schizophrenia, probable schizophrenia, psychopathy, or inadequate personality	
	Biological relatives	Adoptive relatives
Index cases (n=33)	20 out of 156	4 out of 74
Control cases (n=33)	7 out of 150	3 out of 83
Significance (exact probability test)	$p<.01$	n.s.

As may be seen there is a significantly increased prevalence of schizophrenia among the biological relatives of the index cases as compared to the control cases, while there was not an increased prevalence of schizophrenia among the adoptive relatives of the index cases as compared to the controls. This is a result consistent with the genetic but not the psychological transmission hypothesis. Although not presented in the table, most of the schizophrenic biological relatives of the index cases were "borderline schizophrenics" a datum which supplies some additional evidence for the genetic relatedness of this syndrome and process schizophrenia. There were no reactive schizophrenics among the biological relatives of the index cases and furthermore there were no process schizophrenic relatives among the biological relatives of the 6 reactive index cases, a finding which does not suggest a genetic relationship between the two types of illness.[4]

Since the Heston study had shown an apparent increase of other psychiatric illness in the offspring of schizophrenic parents it was logical to inquire if, similarly, there was an increased prevalence of other psychiatric illnesses among the relatives of the index cases. On the basis of Heston's results, the diagnoses of psychopathy, character disorder, and inadequate personality were grouped together with schizophrenia and probable schizophrenia and their frequency among the biological and adoptive relatives was determined. The results are shown in Table 3. As may be seen, there is a small but statistically significant increased prevalence of these disorders among the biological but not the adoptive relatives of the index cases. The very small sample dictates caution but the results are compatible with Heston's finding of increased nonschizophrenic psychopathology in schizophrenics' offspring.

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[4] Nonadoption studies have shown some relationship between the two forms of schizophrenia. The expected rates of process schizophrenia among the relatives of reactive schizophrenics is comparatively low so that the small sample size in this study may have obscured such a relationship.

This study suffers from two weaknesses, one methodological, the other logical. The methodological weakness is that ratings were determined from formally recognized psychiatric illnesses. Since recognized illness constitutes only a small fraction of total illness in the population, the observed prevalence rates are low; they are underestimates. Furthermore, the "control group" may have contained ill members with unrecognized illness since a weak criterion of mental health was employed: lack of formal recognition of illness. If the control group did contain members with unrecognized illness the prevalence rates in their relatives may be inflated (as compared to values for relatives of normal individuals). These methodological problems could be eliminated by interviewing all of the cases and their relatives. This laborious but necessary work is currently being performed.

The logical problems of the study relate to the initial question to be answered, i.e., the fraction of schizophrenics with an apparent genetic background. In this study 27 percent of the index cases had a schizophrenic or probably schizophrenic biological relative, rates which are of the same order of magnitude as those seen among the first degree relatives of nonadopted schizophrenics (Rosenthal, 3). The problem is that without knowing the mode of genetic transmission, one cannot ascertain the expected rates to be seen among the biological relatives. The reason for this is as follows: if the mode of transmission of a trait is not a simple dominant,[5] chance will dictate that in some instances no relative of an index case will possess that trait. If a trait is recessive, for example, neither of the parents (who are usually heterogeneous) will manifest that trait while one-quarter of the sibs will.[6] Accordingly, all that may be said is that the figure of 27 percent represents a minimal estimate of the number of schizophrenics in whom genetic factors play a role.

In none of the studies reviewed was the psychological environment of the adopted schizophrenic evaluated. Psychiatrists who favor the psychological mode of transmission (e.g., 9, 19–23) have clearly described the deviant parenting to which adult schizophrenics have been exposed in childhood. As already mentioned, these studies are difficult to interpret since one cannot determine if the abnormalities in the parent are a manifestation of illness in them rather than the cause of illness in the child.

In order to answer the question of whether a deviant psychological environment is a necessary factor in the genesis of schizophrenia, a third design was employed (Wender et al, 24). In this study three groups of parents were

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[5] Some psychiatric geneticists have argued that the "schizophrenic gene" is dominant but with small penetrance.

[6] One may calculate that if the mode of transmission were that of a single recessive, 60 percent of a three-member sibship (the average members in this study) would manifest the trait.

TABLE 4. Global Severity of Psychopathology Rating Scale

1 Normal—without any disorder traits.
2 Normal—with minor psychoneurotic traits.
3 Psychoneurosis or mild character neuroses.
4 Moderate-to-marked character neuroses.
5 Severe character neuroses, moderate to marked cyclothymic character, schizoid character, paranoid character.
6 Borderline schizophrenia, acute psychoses.
7 Schizophrenic psychosis.

evaluated: the adoptive parents of adult schizophrenics (the "adoptive schizophrenic group"); parents who had reared their own (now adult) schizophrenic offspring (the "biological schizophrenic group"); adoptive parents who had reared normal children (the "adoptive normal group"). Ten sets of parents in each group were evaluated with intensive psychiatric interviews and psychological tests. On the basis of these data, the parents were rated on a global scale of psychopathology (Table 4). The average levels of psychopathology in the three groups is shown in Table 5.

The biological parents group was considerably more disturbed than the adoptive schizophrenic group who, in turn, were slightly but significantly more disturbed than the adoptive normal group. Of interest is the compari-

TABLE 5. Frequency and Severity of Psychopathology in the Three Groups of Parents

Group		Mean psychopathology (±S.D.)
Biological schizophrenic	Fathers	4.2 ± .95
	Mothers	4.9 ± .82
Adopted schizophrenic	Fathers	3.3 ± 1.01
	Mothers	3.5 ± 1.28
Adopted normals	Fathers	2.6 ± .74
	Mothers	3.0 ± .68

Significance, one-tailed t-test:
Adoptive schizophrenic parents vs Biological schizophrenic parents, $p < .005$.
Adoptive schizophrenic parents vs Adoptive normal parents, $p < .05$.
Adoptive schizophrenic mothers vs Biological schizophrenic mothers, $p < .05$.
Adoptive schizophrenic mothers vs Adopted normal mothers, NS
Adoptive schizophrenic fathers vs Biological schizophrenic fathers, $p < .05$.
Adoptive schizophrenic fathers vs Adoptive normal fathers, NS

TABLE 6. A Comparison of the Data of Alanen (1966) and the Adoptive Parents Study

Alanen		Adoptive parents study	
Group	Mean severity of psychopathology	Group	Mean severity of psychopathology
Fathers of schizophrenics	4.8	Fathers of schizophrenics	4.2
Mothers of schizophrenics	4.8	Mothers of schizophrenics	4.9
Parents of schizophrenics	4.8	Parents of schizophrenics	4.6
Fathers of neurotics	3.2	Adoptive fathers of schizophrenics	3.2
Mothers of neurotics	3.4	Adoptive mothers of schizophrenics	3.5
Parents of neurotics	3.3	Adoptive parents of schizophrenics	3.4
		Adoptive fathers of normals	2.6
		Adoptive mothers of normals	3.0
		Adoptive parents of normals	2.8

son of these findings with those of Alanen (19), a Finnish investigator who assessed psychopathology among the biological parents of schizophrenics and neurotics (Table 6). As may be seen, the severity of psychopathology is similar in the two groups of biological parents of schizophrenics while Alanen's parents of neurotics are comparable to the adoptive parents of schizophrenics. The small sample size of the Wender et al study and two serious methodological weaknesses (sampling problems and lack of blindness in the interviewer) again dictate caution in accepting the results. If replicated (and a replicative study is being conducted) this experiment would provide evidence that deviant upbringing is not a necessary condition for the development of some forms of schizophrenia.

Discussion

I would now like to summarize the conclusions that may be drawn from these studies and the questions that remain to be answered.

First, these studies present—for the first time—incontrovertible evidence that genetic factors play a role in the genesis of some of the schizophrenias. Second, these studies have shown that in "average" environments, genetic factors are sufficient to produce the disorder. Third, these studies have shown that deviant psychological treatment is not necessary for the production of some forms of schizophrenia. These conclusions may appear trivial to those who have previously accepted the genetic position, but these studies provide the first unquestionable support for that position. Much psychological research is devoted to documenting the "obvious."

A fourth finding is that of the apparent genetic relatedness between classical process schizophrenia and the borderline schizophrenias. This is a finding of considerable interest since these illnesses appear to be much more common than "classical" schizophrenia and since many psychiatrists consider the borderline schizophrenics to be "pseudo-schizophrenic neurotics" (e.g., Klein & Davis, 25).

A fifth and surprising finding of the Heston and Kety et al studies was the increased prevalence of nonschizophrenic psychopathology among the biological relatives of schizophrenics. If replicated this finding would suggest that the schizophrenia gene or genes play a role in the etiology of a broad spectrum of psychiatric illness. The hoary concept of neuropathological taint may have to be exhumed and reviewed.[7] The chronic difficulty facing psychiatrists in delineating discrete nonoverlapping syndromes, the persistent and perverse tendency of supposedly distinct illnesses to blur peripherally into each other, and the total failures in attempts to elucidate specific etiologies have caused some psychiatrists to abandon the medical model. For example, Menninger (27) has argued that there are no distinct psychiatric diseases and that the seeming qualitative difference in manifest psychopathology are the product of different degrees of psychological regression in response to differing degrees of maladaption. The data reported in the article cannot refute this contention, but they do suggest that the same perplexing state of affairs may be the product of genetic factors alone.

Next, what questions do these studies not answer? They supply no answer to the mechanism of genetic transmission and cannot help to decide whether it is dominant, recessive, or polygenetic. The data likewise cannot explain the basis for the varying manifestations seen in the schizophrenic's biological relatives. The appearance of no schizophrenic illness may be a manifestation of the possession of a few of the polygenes, all of which must be present for the full-fledged schizophrenic illness, or may represent diminished penetrance due to the modifying effects of other genes or certain environments. ("Penetrance," it is useful to remember is a description not an explanation).

Another question left unanswered relates to the psychological trait or traits which are the phenotypic products of genetic transmission. Psychiatrists have proposed that certain psychological attributes are associated with or form the basis for the development of schizophrenia. A partial list of these attributes would include a disorder of thinking (Bleuler, 2), introversion (Jung, 28), increased sensitivity to anxiety (Mednick, 29) and an innate inability to experience pleasure (Rado, 30). An answer may be forthcoming by examining the presence and intensity of these attributes in the nonschizophrenic biological relatives of schizophrenic cases. At the present time, the question remains unanswered.

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[7] This concept is of some antiquity. In *The History of Melancholy* written in 1651 (26), Burton stated ". . . and that which is to be wondered at, it (melancholy) skips in some families and goes to the son, or takes every other, and sometimes even third in a lineal descent, and *does not always produce the same, but some like and some symbolizing disease.*"

Another unanswered question involves the role of experimental factors in the development of the schizophrenias. It is worth reiterating what these studies have not shown about the role of nurture in the development of the schizophrenic syndrome. First, they cannot—for reasons already discussed—provide an estimate of those schizophrenics which are genetically produced; they can only provide a minimal estimate. [Parenthetically, it is worth repeating that the converse of "genetic" is not "psychological." Organic, nongenetic factors have been strongly implicated in the etiology of childhood psychosis (Goldfarb, 31).] Second, these studies have not shown that some forms of schizophrenia are not produced solely by psychological factors. Such an assertion would be hard to document since it would require demonstrating that an individual who became schizophrenic was clear of the schizophrenic genotype, but it might be tested by evaluating the outcome in children born to normal biological parents and reared by schizophrenic parents. Such a study is currently being conducted (Rosenthal, et al, 32).

Third, these studies do not indicate to what degree the phenotypic expression of the genotype may be aggravated or mitigated by the psychological and social environment. As has been mentioned it is possible that offspring schizophrenics raised in adoptive homes are as apt to develop schizophrenic psychopathology as when reared by their natural parents, but that their level of social functioning may be higher. This hypothesis can be tested by comparing adopted away schizophrenic offspring with those reared by their own parents. Such a study is now being conducted.

SUMMARY

Previous research in the etiology of the schizophrenias has implicated both nature and nurture in the genesis of the syndrome, but had been unable to evaluate their relative contributions. Recent studies employing the strategy of studying adopted persons have permitted a partial answer to this question. They have shown that genetic factors are sufficient to produce some forms of the illness, that deviant psychological experience is not necessary to produce the illness, and that genetic factors associated with schizophrenia may produce other, nonschizophrenic, psychiatric illnesses. These studies have not disproved the contention that some forms of schizophrenia are nongenetic (biological or psychological) in origin, nor do they provide an answer

to the question of how much environmental factors may influence the manifestation of the schizophrenic genotype(s).

The strategy of studying adopted persons can obviously be employed to study other psychiatric disorders whose origin, like that of schizophrenia, has long been the subject of relatively fruitless debate. Currently, the method is being used to study alcoholism (Winokur et al, 33), depressive illness (Kety et al, 34), and sociopathy (Schulsinger, 35).

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GENETIC ANALYSIS OF HUMAN BEHAVIOR[1]

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> Each one of us differs from his fellows, not only in bodily structure and the proteins which enter into his composition, but apart from, or rather in consequence of such individualities, men differ in mental outlook, character, and ability.
>
> A. E. Garrod (1)

This remark was made by Sir Archibald Garrod in 1929. It reflects his recognition of the source of human uniqueness in the specificity of proteins and, although at that time nothing was known of the biochemical identity of the genetic material, he knew that the specificity was conferred by the genes. To Garrod, the biochemical, structural, and behavioral attributes of human beings were all expressions of their genetic individuality; structure and behavior were the result of biochemical individuality, and the latter stemmed from the molecular qualities of the chromosomes (2). Since Garrod's time, biologists have gathered information testifying to the validity of his ideas. As a result of the discoveries of the past 15–20 years there have emerged some general and unifying genetic principles, applicable to all life, and which provide insight into metabolism, morphogenesis, natural selection and evolution, and human behavior. It is the purpose of this paper to examine human behavior genetics as it is presented in the literature and in the light of these central genetic principles.

Reviews of behavior genetics generally avoid rigid definitions of behavior, perhaps because they are written for the initiated, but also perhaps because the word has many meanings. One way of dealing with the question is to say that behavior represents a response to some stimulus or other, and that in its broadest definition the response may involve; a molecular reaction within a single cell; the concerted action of many cells within an organ or tissue; or the act of a whole organism or even a population. A more restricted usage, however, defines behavior in its social context and deals with sensory responses, intellectual qualities, emotions, and personality traits.

Genetics is the study of the heritable components of variation, and has to do with the nature of the genetic material, its role in replication and information transfer, and its contribution to the phenotypic diversity of individuals. Genetics possesses a quantitative analytical method designed to assign responsibility for variations to individual genetic units in particular chromosomes, as well as to describe the distribution of genes in families and populations. Behavior genetics, then, consists of the use of genetic analysis to relate genes to behavioral phenotypes.

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A second purpose is that of bringing an important new discipline to the attention of physicians who do not customarily think of their patients' behavior as having any significant genetic origin. That they do not is not surprising, because genetics has been ignored by psychiatrists and other behavioral scientists. Thus, although medical students are aware that the source of much human diversity is encoded in the DNA, their thoughts are too seldom guided to consideration of the relationship of the DNA to human social behavior. At the same time, physicians are being called upon increasingly to deal with behavioral variation; pediatricians to deal with learning disorders, "behavior problems," and mental retardation, while internists and psychiatrists are confronted by psychoses, neuroses, and a wide range of maladaptive responses to disease, life's stresses, and drugs. These are abnormalities and in the traditional medical domain, but there is the additional requirement that physicians be aware that normal behavioral individuality is also conditioned by the genetic endowment.

For these reasons the information will be presented from the physician's viewpoint with the thought that doctors will be increasingly involved in the discovery of their patients' innate capacities and in helping to design environments which will avoid nonadaptive inherent leanings and capitalize on favorable potential.

THE CENTRAL THEMES OF GENETICS

The following is a brief recitation of the central ideas of genetics. Encoded in the DNA is information governing the specificity, the amounts, and the times of appearance in developmental sequences of proteins. Proteins, serving as structural materials, transporting devices, and enzymes, are central to all metabolic functions of the cell and body. DNA replication, which ensures the continuity of species, is sometimes imperfect. The resulting mutations occur randomly but regularly, with frequencies of the order of 1 per 50,000 to 100,000 replications per locus per generation, and they are reflected in changes in the qualities or amounts of the proteins. Mutations are the ultimate source of genetic variation and, together with recombination and the independent assortment of chromosomes, ensure that each individual is genetically unique. Natural selection acts to eliminate extravagant variation and whether or not genes are retained or eliminated depends upon the adaptive condition of their phenotypes. Since the genetic code is universal, the relationships of all species can be visualized in terms of differences of amino acid content of homologous proteins. A number of consequences flow from these central ideas which account for human individuality and diversity (3).

1. Since the genes exert ultimate control over metabolism and homeo-

stasis, and since mutation is random, any function under innate constraint *must* be variable.

2. The randomness of mutation allows for a very large number of variants of each gene product (4, 5). The differences caused by these variants depend upon whether the structural change alters the activity of the protein. Empirical observations suggest that only a few percent of all variants are overtly harmful, but mutations producing slight to moderate changes in activity of a single enzyme may account for differences in rates of metabolic pathways and, when in combination with mutants specified by other loci, could be responsible for variation regarded as "normal," or of differences, and even diseases said to be "multigenic" in origin.

3. The complexity of metabolic networks ensures that phenotypic variation can be due to mutations at more than one locus, as well as to multiple alleles for a single locus. This expectation has been borne out whenever the question is raised (6).

4. Although there may be many variants of proteins specified by genes at a given locus, the number detected with frequencies of 2 percent or more is few, often limited to one, two, or three (7). These genes, called polymorphic, contribute to normal variability and act as a background against which the contrapuntal variation of the rare mutants is seen. Of the latter, each person might have a few or as many as several hundred.

5. The polymorphic genes are found in widely disparate populations all over the world, indicating the genetic kinship of the races. Indeed, it is likely that the sources of intraracial variation are more numerous than those of inter-racial difference. The evolutionary view sees racial classification as of practical social use, but in recognizing that the races are composed of individual persons, each possessing a unique genotype made up for the most part of genes shared by other human beings, it reveals the error of generalizations about racial differences and the folly of claims of genetic superiority.

6. The traditional process to elucidate a gene effect is to trace the path from character to gene by clinical, physiological, and biochemical study, focusing finally on some protein and its inferential gene. We now know of genetically determined protein variants and enzyme deficiencies whose metabolic significance is unknown. We have long been accustomed to phenotypes whose relevant genes are unknown, we are now discovering genes in search of phenotypes (6).

7. The idea that behind every protein is a gene which has specified its structure has simplified the genetic analysis in some respects. The detection of any peptide or protein testifies to the gene which specified it and we may look for differences which we know must exist. It is even possible, in the presence of structural variants of a single protein, to draw the *inference* that they are the products of allelic genes. This does not mean that Mendel's laws of segregation and independent assortment have been repealed or superseded, but that such an inference is justified as a first approximation under conditions in which it cannot be subjected to genetic test (8).

8. There remains one major gap in this otherwise dramatic story; the lack of insight into the undoubted genetic control over morphogenesis and development. It is likely that human "regulatory" genes exist and that they will be shown to resemble those of lower organisms, but although some interesting models have been proposed, descriptions of such genes are lacking (9).

To summarize, it is clear that all aspects of metabolism, development, and behavior are under genetic control and must reflect functional differences imposed by gene variation. If the genotype of each human being is unique, so must be his behavior. This is not to deny or belittle the effects of experience, obviously genes function only in environments, but to say that the genes specify the limits within which nongenetic influences can exert their effects.

Modern knowledge of the biochemical properties of gene action, together with our increasing recognition of the extent of genetic variation, suggest that the future will bring elucidation even of complex multigenic characteristics in terms of the separate functions of each of the genes responsible for the differences (10). What is needed to bring progress in this field is an atomistic point of view toward all forms of genetically determined variation, in which an attempt is made to resolve complex phenotypes into components which can be analyzed for their genetic origin, and whose physiological and biochemical properties can be studied.

GENETICS AND THE BEHAVIORAL SCIENCES

There is no doubt that the behavioral sciences have flourished without benefit of genetic theory or thought. Although Darwin and Spencer made clear the biological and evolutionary elements of behavior and made the study of animal psychology relevant to that of man, until recently little attention was given to the possibility of the hereditary origin of psychological differences between human beings (11) Indeed, even today, apart from the contributions of a relatively small group of behavior geneticists, the literature of psychology and psychiatry reflect little grasp of genetic principles, and even less appreciation of their application. Most journals of both psychiatry and psychology list only an occasional article with any genetic content, and, with rare exceptions, the textbooks offer no more (Table 1). In contrast, 10–15 percent of the papers published in widely read clinical medical journals present genetic subjects, and all modern texts of medicine carry sections on the principles of genetics and inborn metabolic disorders, as well as prominent review of the hereditary aspects of individual diseases.

The reasons for these disparities are no doubt a property of the differences in the aims of the practitioners of these disciplines. Physicians, traditionally concerned with disease in individuals, have not hesitated to apply genetics wherever it could be shown to be relevant. Diseases segregating as Mendelian characters have been known for generations, and in modern times the biochemical elucidation of the pathogenesis of hereditary diseases has often led to means of treatment or prevention. Psychology seems to have a different

TABLE 1. PROPORTIONS OF PAPERS DEALING WITH GENETIC SUBJECTS IN SEVERAL JOURNALS

Journals	1961–1965			1966–1970			1961–1970		
	Genetic Papers	All Papers	% Genetic	Genetic Papers	All Papers	% Genetic	Genetic Papers	All Papers	% Genetic
American Journal of Medicine	106	932	11.37	134	993	13.49	240	1925	12.47
Journal of Clinical Investigation	70	1038	6.74	142	1154	12.31	212	2192	9.67
Pediatrics	136	962	14.14	159	1096	14.51	295	2058	14.33
Subtotal	312	2932	10.64	435	3234	13.41	747	6195	12.06
British Journal of Psychiatry	24	550	4.36	57	808	7.05	81	1358	6.00
American Journal of Psychiatry	19	1226	1.55	18	1084	1.66	37	2310	1.60
American Journal of Orthopsychiatry	5	377	1.32	6	345	1.74	11	762	1.44
Psychosomatic Medicine	2	178	1.12	5	268	1.87	7	507	1.38
Subtotal	50	2331	2.14	86	2505	3.43	136	4937	2.75
Psychological Reviews	1	207	0.48	1	226	0.44	2	433	0.45
Psychological Bulletin	3	277	1.10	4	365	1.10	7	642	1.10
American Psychologist	0	205	0.00	1	320	0.31	1	525	0.19
Subtotal	4	689	0.58	6	911	0.66	10	1600	0.63
Behavioral Science	1	182	0.55	5	219	2.28	6	401	1.50
Behavioral Research and Therapy	2	114	1.75	0	217	0.00	2	385	0.52
Subtotal	3	296	1.52	5	490	1.02	8	786	1.10

mission; to describe behavioral patterns and mechanisms for the whole species. In this context, heritability is important in differentiating one species from another, and in consequence, individual variations due to major gene effects are regarded as a nuisance and experiments are designed so as to minimize them. Even the word "genetic" is used in a different way; genetic psychology means developmental psychology, and Piaget, in some aspects of his work, observed only a handful of growing children. The behaviorists, even while acknowledging hereditary control over individual differences in structure, concluded that behavioral function was subject only to the conditioning influences of experience (12). Watson's famous statement that experience could make of any person a "doctor, lawyer, artist, merchant chief and, yes, even beggar-man and thief, regardless of his talents, penchants, tendencies, abilities, vocations, and race of his ancestors" is an example of thinking unconstrained by biology or genetics, and it is one which turns up in modern guise even today. It says that all members of a species (or race) are essentially the same, but open to wide variation induced by experiences. The important genetic differences, in this view, are those between the taxonomic groups. This attention to species patterns also caused psychologists to focus on rather broad behavior forms such as intelligence, learning, perception, memory, and so on, all phenotypes which would be most unlikely to reveal the kind of discontinuities which engage the attention of the geneticist, and which, it should be added, have proven awkward to study as such from the biological and genetic point of view.

Psychiatry has been always more biologically oriented, dealing as it does with abnormal behavior, and today many psychiatrists see mental disturbance as a response of the whole body to life experiences. But, although the biochemical attributes of the psychoses are increasingly emphasized and studied, there are few psychiatrists who reason that particular persons respond in individual ways to specific environments because, in addition to past experience, their innate endowments predispose them to do so. It is the life experiences which are emphasized and the limiting, determining, influence of the genetic constitution is usually ignored.

BIOMETRY AND THE BEGINNINGS OF BEHAVIOR GENETICS

Darwin and his contemporaries were only peripherally interested in differences between individual human beings. It was Darwin's first cousin, Francis Galton, who was the first to study systematically the hereditary causes of human variation. He observed individual differences in such sensory and psychological functions as; mental imagery, word associations, memory, hearing, and smell, and the distributions of these characters in families were traced. He measured everything and divided quantitative data into quartiles, deciles, and so on, working out scores and distributions for such characteristics as human beauty and the therapeutic skills of physicians, suggesting for the latter that the score determine the compensation. He was also the first to use twins to differentiate the effects of nature and nurture.

His quantitative approach led to the invention of the correlation and regression coefficients. Correlation coefficients were calculated for measurements of characteristics in sib pairs, parent-child pairs, and in pairs of persons of other degrees of relationship, and the observation was made that on the average, measurements for children tended to regress from those of their parents toward the population mean. These methods are still in use and form the basis for much of the work in the genetic analysis of behavior today.

Apparently Galton's mathematics was rather weak, but this deficiency was more than made up by his devoted follower and biographer, Karl Pearson, who, it should be noted, was a mathematician, not a biologist. Pearson undertook to test Galton's ideas of heredity by refining and extending the biometric techniques, and he assumed the defense of the biometric viewpoint when it came under attack by the Mendelists soon after 1900 (13). The Mendelists, led by the biologist Bateson, insisted that inheritance was discontinuous, that the hereditary factors segregated, assorted independently, and showed dominance. The differences were debated with heat and acrimony for several years, but in time it became apparent that the two sides were but the poles of a spectrum and the argument subsided.

Although in time it was agreed by all that the Mendelian genes are the source of all genetic variation, subsequent study of the heredity of discrete, segregating differences on the one hand, and of continuous variation on the other, was based on divergent viewpoints, was carried out with different methods, and seldom by the same people. The former path led, through increasing refinements in the definition of the gene, to the discovery of its biochemical nature and to the description of the genetic code. The latter, through increasing refinement of the mathematics involved, led to the detection of the extent of the hereditary contribution to such broad phenotypes as those encompassed by behavior as well as of others of importance to animal and plant breeders. It could hardly have been otherwise, since the different phenotypes could not have been analyzed by the same methods.

The genetic analysis of behavior proceeded by both paths, but with unequal success. The pedigree method was taken up by enthusiastic investigators who largely misused it. For example, Davenport published family studies in which such characters as general bodily energy, musical ability, literary composition, and pauperism were treated as if the differences in the members of the families were due to single genes. This oversimplified concept of the cause of variation in behavior was taken up in the 1920's by the eugenists who used it to prove the undesirability of the poor, of racial minorities, and of whomever else they didn't like.

As for the analysis of continuous variation, we have seen that Galton and Pearson gave it a good beginning. Pearson continued with the study of the inheritance of behavioral traits for a time, but he was hopelessly nonbiological and in time reverted to mathematics. He was also an eugenist, and it is to be feared that his studies of the inheritance of personality gave ammunition to the environmentalists who denied any heritable influence on behavior at all. Pearson's methods for the study of metrical variation were extended by Fisher, who invented the analysis of variance and the heritability coefficient, by Wright and later by Mather, who used the term "polygenes" to designate the genes contributing to continuous variation.

From the beginning the biometricians were less interested in the qualities and effects of the genes themselves than in the analysis. By calling them polygenes and thinking of them as numerous and additive, they could dodge the issue of how the genes were able to account for the variation which the mathematical methods were designed to measure. Later, corrections were introduced into the analytic method to include such consequences of Mendelism as segregation, dominance, and assortative mating, but despite these concessions to biology, the biometricians continued to emphasize the mathematical treatment of continuous variables. Although this indifference to the numbers, kinds, and effects of the genes does not prevail today, the usual approach to human behavior genetics continues to be mathematical (14, 15), and is occasionally hostile to the Mendelian method (16, 17).

Animal behavior.—Genetic analysis of the behavior of animals has proceeded more rapidly than that for human behavior, mainly because of the ease of genetic manipulation of animals and the opportunity for physiological and biochemical study. Such investigations are of importance for their illumination of genetic and psychological principles, but they are also of interest to the human biologist because they provide useful models to be copied in concept if not in detail. The relevance of animal models in concept and method is especially well demonstrated in the excellent book of Fuller & Thompson (18), as well as in many more recent symposia and reviews (19–22).

For example, Hirsch has localized genes contributing to geotactic behavior of *Drosophila* to particular chromosomes (23). The methods he used are not applicable to man, but the observations demonstrate the reality of multigenic control of a complex behavior. Several investigators have used genetic, neurophysiologic, developmental, and biochemical methods to describe audiogenic seizures in mice and to relate them to a few, still elusive, genes (24), while Rodgers and McClearn have studied the behavioral, nutritional, and biochemical aspects of the affinity for, and aversion to, alcohol in contrasting inbred mouse lines (25). It is interesting that the two most polarized lines differ markedly in the activities of alcohol and acetaldehyde dehydrogenases (26).

Perhaps the most elegant and most conceptually illuminating studies are those of Benzer and his colleagues (27–30). Their idea is to dissect a complex behavioral phenotype into its components using single gene mutants as the dissecting instruments. The behavior is phototaxis; the organism *Drosophila melanogaster.* Flies normally move toward light unless visual perception is impaired. The questions the investigators ask are, what are the molecular, biochemical, and neurophysiological mechanisms or steps in achieving phototactic responses. The answers should be attainable by the study of genetic mutants in which one element is altered at a time. The procedure involves discovery and characterization of phototactic mutants after treatment of flies with a mutagenic agent. The abnormal flies are then bred and genetic mapping reveals that some of the mutant genes are alleles, while others occupy different loci. Flies representative of each are then studied anatomically and physiologically. Several mutants reveal anatomic variations in the visual system. Others show differences in the electroretinogram (ERG). One of these shows near absence of potential, while others have characteristic changes in the record, one also showing change in body pigmentation. Two more combine changes in the ERG and age-dependent degenerative changes in the receptor cells, and, of course, there are some whose ERG records are normal. All of these types of defect represent gene mutation at different loci, and there are, in addition, alleles for each. Presumably, if the alleles could be further characterized, they, too, would be found to be heterogeneous. The method, carried to its logical conclusion, could uncover hundreds of genes which, operating through their immediate molecular products, exert effects upon phototaxis as well, no doubt, as upon other phenotypes to which they are also related. Furthermore, it should be possible to characterize some, possibly many, in molecular terms and even to study the properties of developmental differences, since the developing visual systems of larvae of strains known to possess the mutant under scrutiny could be examined. Other studies have disclosed mutations elongating, shortening, and obliterating a circadian rhythm in flies (31). In this instance, all three mutations appear to affect the same gene.

The end product of such investigations is knowledge of the numbers, qualities, and functions of the genes involved in complex multigenic systems. The human parallel is immediately evident, and while we can hardly expect the rapid progress which is open to the *Drosophila* investigators, it should be possible to approach human multigenic phenotypes with this atomistic viewpoint. Obviously, we cannot create new mutations in man, but they are undoubtedly there in the population, awaiting discovery. Perhaps the main obstacle to the application of this method to human material lies in our inability to map the genes so as to decide between allelism or separate loci, but the methods are developed and we are certainly on the verge of an exponential rise in the rate of discovery of new linkages (32).

GENETIC ANALYSIS OF BEHAVIORAL PHENOTYPES

INTELLIGENCE, MENTAL ABILITIES, AND PERSONALITY

These qualities are especially awkward for genetic analysis. They are difficult to define, to test comprehenisvely, and to score, and they are continuously distributed characters. They are, however, of great importance to behavioral scientists, to educators, and to scoiologists, and as such have been the object of much study (14, 15, 19, 21, 22, 33, 34). Many of the investigators are psychologists who have attempted, by mathematical methods, to discover whether or not cognitive or personality functions are hereditary and the extent to which the variance of scores of appropriate tests is due to genetic or environmental causes. To do this the methods of correlation and factor analysis have been used for test scores of twin pairs (34). The argument runs that the variation for test scores within monozygotic twin (MZ) pairs must be nonhereditary, since the twins are genetically alike, while that within dizygotic (DZ) pairs must be both hereditary and due to the special experiences of each of the twins. The latter, embracing both kinds of difference, should be larger than that for the monozygotics, and some sort of comparison of these two values should provide a measure of the extent of heritability of the character in question. An additional measure can be obtained by comparing results of MZ pairs reared together with those reared apart. Since the twins reared apart will have experienced unlike environments, the degree of likeness of their test scores will be an expression of the effects of their common endowment. Factor analysis of intrapair correlations of scores of several tests can be carried out to determine the degree to which the tests are characterizing independent attributes, or whether they are simply different ways of testing the same thing.

Intelligence.—Psychologists would like to know whether or not and to what degree intelligence is heritable. The methods alluded to above have been employed to this end, using as measures of intelligence a number of I.Q. tests. The results of 52 such studies were summarized in 1963 by Erlenmeyer-Kimling and Jarvik and these are shown in Table 2, in which observed and expected correlation coefficients for pairs of relatives and unrelated persons are compared, assuming multigenic inheritance (35). Heritability quotients calculated for most of these studies run from .60 to .80, indicating that as much as 80 percent of the variance in I.Q. test performance

TABLE 2. MEAN CORRELATION COEFFICIENTS FOR I.Q. TESTS

Pairs	Observed	Expected
Parent-child	0.50	0.50
Sib-sib, reared together	0.49	0.50
Dizygotic twins	0.53	0.50
Unrelated, reared apart	0.01	0.00
Unrelated, reared together	0.23	0.00
Foster Parent-child	0.20	0.00
Monozygotic twins, reared together	0.87	1.00
Monozygotic twins, reared apart	0.75	1.00

Adapted from Erlenmeyer-Kimling & Jarvik, Ref. 35.

could be genetic. This does not mean, however, that 80 percent of each person's I.Q. performance is dependent upon his endowment; it is an average applicable to the samples tested and not necessarily to all human beings. Indeed, many investigators believe that heritability coefficients represent an oversimplification of conditions, the details of which are only dimly perceived (36). At the same time, and despite some valid criticisms of the methods used, scarcely anyone would fail to acknowledge that the data do indicate a degree of genetic influence on performance of I.Q. tests and, by inference, on at least some aspects of intelligence.

There has been some question of precisely what the psychometric tests measure, more particularly, if they do measure intelligence whether that is a single, general ability underlying all of the tests, or whether it is a composite quality embracing many separate abilities. The question was first answered by Spearman, who proposed a general intelligence factor which was measured in varying degree by all intelligence tests. Still another answer was offered by Thurstone, who factored out several "primary mental abilities" which could be measured separately. These are verbal, space, number, word fluency, reasoning, and memory. Separate intellectual capacities are also implicit in the Wechsler tests with their verbal and performance divisions, each with several subtests. Further characterization is being carried out by Guilford, who sees intellectual ability as divided into five "operations": cognition, evaluation, memory, convergent production (deductive reasoning), and divergent production (inductive reasoning) (37, 38). Each contains figural, symbolic, semantic, and behavioral elements, and each can be further characterized in six additional "product" categories as units, classes, relations, and so on. Thus, in Guilford's scheme there are 120 intellectual attributes or functions. A clue to the validity of this scheme will be the invention of uncorrelated tests appropriate for each of the functions. Guilford and his colleagues have devised new tests or adapted old ones for many of the boxes in the matrix, but not for all. Vandenberg has shown some heritability for some of the primary mental abilities, for several of a small sample of Guilford's functions, and for some Wechsler subtests (15, 39).

In summary, intelligence is defined by scores in performance tests which psychologists say test intelligence. Several, or many, separate intellectual functions are suggested as a result of mathematical analysis of several or many scores together and the composite quality of intelligence shows a high degree of heritability, and so do some of the special mental abilities.

All of this is inherently reasonable; surely, the powers of cognition, memory, and reasoning, for example, are intellectual functions and are representative of intelligence. But the separate components of intelligence would be given more substantial identity by the discovery of some anatomical or physiological property or by a direct relationship with some definable genes.

The fact of heritability of the intellectual qualities measured in the tests is of itself evidence of their biological origin, but it says nothing of what the biological attributes are, or of the nature of the genes inherited. This is a source of concern to most behavior geneticists, but some adhere to the view that the aim of behavior genetics should be to characterize the complex, comprehensive phenotypes of psychological and social importance, even though to do so makes it impossible to learn anything about the numbers and functions of the genes which condition them (16, 17, 40). The opposing view suggests that to fail to try to characterize the genes is to be brought to a full stop in understanding behavior (41–43). These differences in attitude appear to reflect the primary orientation of the workers, psychological on the one hand, biological on the other.

The molecular geneticist would sympathize with the latter view. The biometrical method tells us that intelligence and its components are heritable qualities, and it provides us with some useful predictive information for counseling, that is, bright parents are likely to have bright children and vice versa, but it does not tell whether or not a particular character is inherited in a particular family. The biometrical method has "factored out" many intellectual attributes which, because all behavior is mediated by biochemical events going on in structurally organized cells, must be strongly influenced by the genes. Mutation being what it is, variants must exist to account for a large part of the variation of the attributes. The job at hand is to characterize those variations, and since the breeding test is not open to us, it must be by psychological and physiological study of individual differences in single persons and their families. The number of loci containing the "genes for intelligence" may be very large, and the common alleles accounting for the normal range of scores in tests of intellectual function may not produce easily detectable Mendelizing characters, but liberally sprinkled throughout the population there must be genes for each locus, some rare, some common, which an appropriate test could detect, after which the familial distribution could be traced. The test is all-important. Psychometric tests seem to be devised to discover a range of responses under standard conditions. What is needed to discriminate genetic differences are tests sharply focused on narrowly defined attributes and which detect limiting conditions, analogous to the tolerance tests used in metabolic diseases or the selective media used by microbial and somatic cell geneticists.

It is unlikely that biochemical attributes of normal human intelligence will soon be discovered. For example, the genes which determine the number and migration of neurons and the extent and complexity of their interconnections must condition intellectual function, but there is no way to test for them. Probably for some time to come the study of neurological and biochemical correlates of learning will be confined to animals (44–46). Despite these limitations an interesting, long-range approach is being tried by Cohen, Omenn & Motulsky, who are attempting to characterize polymorphic genes specifying human brain proteins (47). When a large number of such proteins are known, it may be possible to correlate protein profiles with behavioral characteristics.

Personality.—Personality factors are even more resistant to genetic analysis than intelligence (48–50). The phenotypes are more elusive and indefinable and undoubtedly even more influenced by the special experiences of the subject. In general, the methods are the same, that is, MZ twin pairs are contrasted with DZ pairs for scores in tests of such characteristics as sociability, social pressure, responsibility, tolerance, and many others. Again, heritability estimates reveal the expression of innate factors, but these estimates are generally lower than those for intelligence (48). This is perhaps not surprising, since the phenotypes and tests are defined altogether in social and pyschological terms. Again, it is proposed that the characters are multigenic in origin and nothing is known about the genes.

Perhaps personality factors are unlikely phenotypes for genetic analysis. Something more useful might come from looking at personality characteristics of persons known to carry such genes as those of the inborn errors of metabolism. It might be useful also to look at intrafamily distributions of personality characters known to be associated with such disorders as coronary artery disease (51) or gout (52). If hyperuricemia really is correlated with performance in I.Q. tests and with social and business success, it would be useful to have data on such associations in large families, especially involving collateral relatives.

Psychotic Behavior

In previous pages the genetic variation of species characteristics has been considered; everyone has some intelligence and some sort of personality. We turn now to phenotypes shared by a limited segment of the population and therefore more likely to yield to tests of genetic origin. The literature of the genetics of psychoses is large and conflicting and, although it compels acceptance of an important role for the genes in their pathogenesis, there is little evidence that genetic analysis has yet contributed much to the classification of the disorders.

The imprecision of diagnostic criteria for both schizophrenia and the affective disorders creates problems of classification and analysis. As Eisenberg put it, "we feel we know what schizophrenia is, even though we find it hard to agree on a definition" (53). The diagnoses are historical and subjective, and lack quantitative physiological and biochemical attributes, so, lacking these "handles," the search for causes, whether genetic and biochemical or environmental and psychodynamic, is handicapped. This lack of markers has also meant that investigations into cause usually reveal the effects of the biases of the investigator whose genetic or environmental preferences are reflected in the way the study is carried out.

Schizophrenia.—The incidence of overt schizophrenia in the population is about 1 percent. Among the parents of randomly selected cases it varies from 2 to 10 percent, depending upon the study; among sibs, 6–12 percent if neither parent is schizophrenic and rises to 8–33 percent if one parent is affected (54). If, in addition to overt schizophrenia, persons showing schizoid-tendencies are included, these figures rose to 44 percent for parents, 46 percent for sibs, and 49 percent for children of schizophrenic index cases (55). Twin comparisons have been reported also, and concordance for MZ pairs varies around 40 percent, while that for DZ twins ranges from 2 to 15 percent (54, 56, 57). Some of the differences between the studies were undoubtedly due to diagnostic criteria, sampling procedures, and so on, but in one study the concordance rate was shown to vary directly with severity, achieving a level of 79 percent for the MZ twins and 45 percent for the DZ twins (54, 56).

These incidence figures, collected with much effort, have been criticized for failing to distinguish environmental from genetic causes of schizophrenia. For example, concordance rates are higher for: DZ twins compared to sibs, like-sex twins and sibs than unlike pairs, and female relatives as opposed to male (54). These observations suggest that rearing in a common environment may have some causal significance. To overcome these objections, studies have been done of persons reared apart from their biological relatives (54, 58–61). These investigations have been carried out with strict attention to experimental detail, especially to elimination of bias in diagnosis. In one study, the incidence of schizophrenia and allied disorders in the biological relatives of schizophrenic persons raised by foster parents was compared with that among the adoptive relatives, and with those for matched controls, also adopted (54). The incidence of the disease among the biological relatives of the schizophrenic adoptees was 2–4 times greater than any of the other groups.

In another type of study, the incidence of schizophrenic syndromes in the adopted children of schizophrenic mothers was compared with adopted controls who were the offspring of normal parents. In one of these studies, one third of the adopted children of schizophrenic mothers showed schizophrenic manifestations, while only one of the 47 controls showed borderline symptoms (54). In a second, 5 of 47 adopted children of affected mothers contrasted with none of the 50 controls (60).,

These results indicate that genetic factors make a significant difference, but they ignore precipitating environmental factors. Accordingly, a study was done in which the psychological status of adoptive parents of schizophrenic children was compared with that of the children's biological parents and with a suitable group of adoptive parents of normal children (62). While there were no overt schizophrenics among the adoptive parents of schizophrenics, there was significantly more psychopathology among that group than among the adoptive parents of normal children. This suggests that severe psychopathology in the household is not necessary for the transmission of schizophrenia, but that some such factors may play a part. Alternatively, it might be that the psychological aberrations in the adoptive parents were induced by the presence in the household of the schizophrenic child.

Further attempts to partition the genetic and cultural contributions are in progress (58). One study compares the incidence of schizophrenia between groups of offspring of nonschizophrenic parents raised by schizophrenic adoptive parents and children of schizophrenic parents raised by nonschizophrenic adoptive parents. Another study will observe the incidence of disease among children whose biological parents are schizophrenic and who are raised by schizophrenic foster parents.

These investigations are presented here in detail because they illustrate the elegance and complexity of experimental design which, given organization and energy, can be worked out to examine the degree of contribution of both genetic and cultural influences to a human behavioral character. They also attest to the need for labor-saving biochemical and other traits which could serve as markers for genetic analysis.

Hypotheses to account for the genetic transmission of schizophrenia abound; mendelian recessive with reduced penetrance, monogenic with modifiers, two loci, and multigenic (63–65). This multiplicity of alternatives is the result of heterogeneity, genetic or environmental or both, but from the molecular point of view there is not much difference between them. When one is able to describe the effects of each gene in molecular terms, penetrance and dominance disappear while multigenic phenotypes are seen to be the compound result of two or three or of many monogenic effects. When it comes to a single case of schizophrenia, the phenotype may be equally the result of a deficiency of a single step in a metabolic pathway, or of deficiencies in several steps of the same pathway, each the result of a mutant at a different locus. In each example, whether or not each gene is exhibiting a codominant, dominant, or recessive effect and showing penetrance or lack thereof, depends upon the means used to measure and describe the effect. To make distinctions between these alternatives or others, one needs biochemical markers.

Such markers are wanting in schizophrenia. Biochemical investigation has focused on the metabolism of the neurotransmitters dopamine and norepinephrine (66–70). One hypothesis proposes an abnormality of transmethylation in which methyl derivatives of dopamine and norepinephrine act as psychotogenic compounds (67). Another hypothesis suggests 6-hydroxydopamine as the culprit (71). Schizophrenic manifestations are also seen in persons who abuse such drugs as L.S.D. and amphetamines, and in persons with chromosomal abnormality or one of several inborn errors of metabolism. It will be important to establish whether these latter are precipating agents in persons possessing a standard schizophrenic genotype or whether schizophrenia is the result of genetic differences in many diverse metabolic pathways. In either event, since schizophrenia is a common disorder it may turn out, for the most part, to be due, not to uncommon genes contributing to marked deficiency of enzyme defect, but to particular sets of rather common genes which are of themselves harmless, or perhaps even selectively adaptive (72, 73), except in those particular combinations.

Affective disorders.—The literature of the affective disorders also reveals diagnostic imprecision and overlap and, again, there are no physiologic or biochemical markers which might be used to distinguish the diseases and their variants. On the other hand, the genetic method has been used with some success to distinguish mania and depression (bipolar disease) from depression alone (monopolar disease), since Perris showed that the affected relatives of bipolar index cases always had bipolar disease, while the affected relatives of persons who had depression without mania had unipolar disease (74). A similar conclusion was reached by Winokur and his colleagues, although they did find some monopolar cases among the relatives of bipolar patients (75–78). These observations, together with the earlier age of onset of the bipolar cases and the significantly higher incidence of disease among their relatives, have caused many investigators to treat these as different entities (54).

The incidence of depression in the general population is about 1 percent. The incidence for relatives of bipolar cases ranges around 15 percent and for monopolar patients from 5–15 percent (54). When at least one parent is affected, these frequencies rise to 20–25 percent and 13–17 percent, respectively (54). Twin comparisons reveal the expected differences with concordance for MZ twins 3 to 20 times greater than DZ twins without distinction as to type (54).

Everyone agrees that there are more females with depressive diseases than males, and the familial distribution of the sexes of affected persons has been taken by Perris and Winokur to suggest sex-linkage for one of the types of the disease, but unfortunately each draws this conclusion for a different form. Perris finds no male to male transmission for the unipolar disorder (79), while Winokur finds equal numbers of males and females among the offspring of affected fathers (80). On the other hand, Winokur's data show a marked deficiency of affected males among the sons of affected bipolar fathers, a category in which Perris finds equal numbers of males and females. There seems to be no way of reconciling this difference, unless the investigators are dealing with genetically distinct groups—an unlikely probability. Winokur has looked for evidence of linkage of bipolar disease with color blindness and the Xg blood group (76, 80). The results are equivocal, but do represent, not only a knowledgeable effort to establish a mode of inheritance for that type of disease, but also a good test of the hypothesis that the condition is genetically determined at all. In general it can be said that for the affective disorders as for schizophrenia, most of the proposed modes of inheritance—monogenic X-linked, autosomal dominant, X-linked and autosomal, multigenic, and so on—represent hypotheses for which no rigorous tests suggest themselves and are, therefore, to be taken as tentative.

Biochemical studies of affective disorders have also focused on the neurotransmitters, including both the catecholamines and serotonin, some emphasizing the former, others the latter (81, 82). Again, deficiency of one, or relatively small decrements in activity of several, of the enzymes in the synthetic or disposal pathways could result in the disease. In addition, evidences of clinical and genetic heterogeneity suggest biochemical heterogeneity. It has been suggested that some depressed people are deficient in catecholamines, while others lack serotonin, and there is thought to be a small group in which the deficiency is primarily of dopamine rather than of norepinephrine (82). Since the specificity of drugs used in treatment is probably related to the specific genetic type, matching of clinical and genetic characteristics has importance in the management of patients in each repeated episode of illness and for any relatives who may later become ill.

INDIVIDUAL DIFFERENCES IN RESPONSE TO DRUGS AFFECTING BEHAVIOR

The influence of nurture on nature is clearly exemplified in the individuality of response to drugs. Genetic theory predicts such individual responses, since drugs act by influencing metabolic processes, often at specific enzymatically controlled points. In addition, the drugs themselves are transported, cross membranes, and are metabolized, all processes capable of exhibiting genetic variations expressed as differences in pharmacologic action.

Tranquilizing agents and antidepressants have revolutionized psychiatric treatment, while other drugs are used widely and illicitly for their euphoric effects. All persons do not respond uniformly to each of these drugs, but for most, little is known of the causes of the differences.

Drug treatment of psychoses.—Some depressed patients respond well to monoamine oxidase inhibitors while others require the tricyclic antidepressants. Pare and his associates studied the response of depressed relatives of patients treated successfully with each type of drug and showed that related persons always responded to the same substance, but not to the other (83). These investigators concluded that since the pharmacologic action of the two types of drug is presumed to be different, the differential response suggested genetically and biochemically diverse causes of the depression. Nortryptyline, a tricyclic compound, produces side effects which are dependent upon the blood level, but not the dose of the drug (84). Investigations of families and twins revealed that the differences in these blood levels are genetically determined (84). Phenelzine, another antidepressant, is excreted as an acetylated derivative. Acetylation of many drugs is known to be controlled by polymorphic alleles which promote rapid or slow acetylation, and Evans and his colleagues have shown that among a group of patients who showed severe side effects from the drug, all were slow acetylators (85).

The phenothiazines are the drugs of choice in the treatment of schizophrenia. A number of side effects are known, including Parkinsonism, dystonia, or weakness and apathy. Ayd reported that twice as many males as females showed the dystonic response, while twice as many females as males manifested the Parkinsonian syndromes (86). Kurland and associates suggested that patients who express the Parkinsonian symptoms are genetically susceptible to Parkinsonism and predicted that there would be more victims of that disease among their relatives than among the relatives of similarly treated patients who did not have the toxic manifestation, a prediction which was borne out by observation (87).

Other drugs.—Reserpine is known to reduce levels of biogenic amines in the brain and these compounds are thought to be low in the brains of depressed patients (81). This drug causes depression in some patients in whom it is used to treat hypertension (88, 89). Quetsch and others reported that 53 of 202 such patients became depressed, in contrast to 9 of 185 hypertensive patients receiving no drug therapy (89). Of the 53 patients, 21, or 10 percent of the total 202, were sufficiently depressed to require psychiatric care or hospitilization. Eleven of these 53, or about $\frac{1}{5}$, had had previous depressions not associated with reserpine treatment, but the most severely depressed patients had had no previous psychotic episodes. Evidently, reserpine causes depression in some patients and not in others; and it would be of especial interest to know how many such depressed patients have relatives who have suffered similarly, though not necessarily due to reserpine.

Amphetamines are much abused drugs which, in some users, produce acute paranoid episodes (90). Reports indicate that a significant proportion of the users have personality problems to begin with and come from families in which parents and sibs suffer from alcoholism and other psychiatric disorders (91). It is not clear, however, whether the incidence of psychiatric disease among relatives of amphetamine users who become psychotic is greater than that among the relatives of users who do not show such toxic effects. Such a study contrasting the incidence of paranoid schizophrenia specifically as well as of other personality deviations might be of genetical interest.

L.S.D. also causes psychotic reactions in some users, and the incidence of such reactions may be heightened among persons who have had schizophrenic episodes (92) and among their apparently normal relatives (93).

Little attention appears to have been paid to the possibility of genetic susceptibility to tolerance and addiction to narcotic and other drugs, or to resistance to addiction. While most investigators deny that there is an "addiction-prone" personality (94), Dole suggests that tolerance and addiction are the result of biochemical specificity (95, 96). If that is true, and if some molecular hypothesis for tolerance and withdrawal such as that proposed by Goldstein & Goldstein (97, 98) should be validated, then some persons should be more easily addicted to drugs than others, and some should be resistant. Goldstein's hypothesis assumes inhibition by the drug of activity of an enzyme under feedback regulation by some neurohumoral end product. Inhibition by the drug promotes increased synthesis of the enzyme which, in turn, requires more drug. Withdrawal symptoms are produced by removal of drug inhibition which permits the full activity of the increased amount of the enzyme. Genetically determined variations in the enzyme, or in the means for its production, could easily enhance tolerance or make it unlikely. There are other hypotheses to explain tolerance, but all presume cellular adaptations which must be subject to genetically determined variations which could account for differences in susceptibility to addiction (99). That some persons may be resistant, even to large doses of heroin, is suggested by observation of the effects of drug withdrawal on babies born to addicted mothers. Zelson and associates report no withdrawal symptoms in about one quarter of infants of mothers who were heavy users (100).

SOME OTHER LIKELY POSSIBILITIES

In the following paragraphs attention will be drawn to some human behavioral traits which are worthy of efforts to characterize genetic variation. Some of them have been considered in this light already, while for others such a cause of differences, or even the differences themselves, have been ignored. The premise from which the investigation should begin is that all behavioral functions, mediated as they must be by the metabolic machinery of the central nervous system, must show genetic variation.

Reading disability.—Variations in ability to learn to read are frequently observed wherever educational opportunity is extensive. Some of these are due to defects of vision or hearing, or to brain damage, or to overriding emotional factors, but probably the most common cause is inadequate schools. But there are some persons who cannot learn easily to read, despite good intellectual endowment and none of these handicaps. Space is insufficient to give more than an abbreviated list of the manifestations of reading disability (101–104). These include; reversal and translocation of letters and syllables; omission, addition, and substitutions of letters, sounds and words; mirror writing and reading, and deficiencies in spelling. Tests may reveal defects in space or directional orientation, in visual or auditory memory, or in integration of visual and auditory perception. There are often marked differences in verbal and performance scores in the Weschsler I.Q. tests, although the full score I.Q. may be normal or high (105). In general, these handicaps are thought to be the result of defects in the processing of visual or auditory information or in the coordination and integration of both (106). Many or all of these expressions are seen in beginning readers, but in the person with reading disability they persist, sometimes for months or years, sometimes forever.

All of these expressions are seldom aggregated in single persons and investigators have been on the lookout for clusters of manifestations which suggest syndromes. For example, Johnson & Myklebust have differentiated visual and auditory types (107). Boder described three groups; one defective in phonetic skills, one in visual perception, and a third with deficiencies of both (108). Kinsbourne & Warrington characterized two groups; one with low verbal scores in the WISC, the other with low scores in the performance subtests (109), while Ingram described three types; one with visuo-spatial problems, a second with difficulty integrating visual and auditory stimuli, while a third has trouble in comprehension of words or sentences and in recall of words to express ideas (110). Whether or not there is overlap in these syndromes is not clear; probably there is, but although familial aggregations of cases are described in the families of most of the types, there are no speculations as to genetic heterogeneity.

The evidence favoring genetic origin of reading disability consists of pedigree analysis and twin studies. Although for 80 years "congenital word blindness" has been said to be hereditary and although many papers cite pedigrees based on hearsay evidence (111, 112), there has been only one study in which parents and sibs were actually examined and tested. This study was done by Hallgren in 1950 and, while it lacks the range of diagnostic tests, the rigor of experimental design, and the genetic insight which might be employed in studies of today, he did discover 160 secondary cases in the families of 116 retarded readers and concluded that "primary dyslexia" is an autosomal dominant character (113).

Twin studies add substance to the idea of heredity. Of 17 pairs of single ovum twins, all were concordant for reading disability, while only 12 of 34 DZ pairs were concordant (114). Other evidences favoring genetic origin consist of the persistence of the disorder in some persons throughout life (115, 116), and a male:female ratio of up to 4 or 5 to 1 (101). To account for the latter, Sladen has suggested an autosomal sex-limited gene which exerts a dominant effect in males and a recessive in females (117).

In summary, there are reasons to believe that some forms of reading disability may be inherited, and clinical differences indicate some genetic heterogeneity. The position may be analogous to that of Benzer's phototaxic variants in *Drosophila* (27–30). Reading is a complex accomplishment involving many different neurological, physiological and biochemical functions, the result of many pathways, blocks in any one or several of which could cause disabilities of varying degree and kind. Thus, it seems likely that some of the genetic causes of reading problems will prove to be multigenic while others will be monogenic. Further investigation calls for study of whole families, using special methods to test parents who have compensated and learned to read, and for characterization of the varieties by clinical, physiological, and eventually perhaps by biochemical means. For the former, tests must be invented which will break through adult subjects' reading technique, exposing the deficiency they have learned to circumvent. For the latter, tests of cognitive and perceptual functions critical to reading, and neurophysiological methods should be used. As an example, Connors has shown abnormalities of cortical electroencephalographic responses in a father and four offspring, all seriously handicapped readers, while the records of the good readers in the family were normal (118).

Biochemical differentiation will be more difficult, but not all inborn errors produce profound intellectual impairment, and the possibility that some may interfere with the development of reading has not been exhaustively explored.

Memory.—It has been suggested that there are two memory phases, short-term and long-term, each with distinctive properties and mechanisms (119, 120). The former is thought to be electrical, although some suggest a molecular change at synaptic junctions, but there is general agreement that protein synthesis is involved in long-term memory storage and retrieval (45). The latter is supported by experiments on animals in which RNA and protein synthesis are observed after learning, and memory retention is prevented by drugs which stop synthesis of protein (45, 121–123). Efforts have also been made to determine whether or not there are qualitative changes in the RNA and protein species which increase in learning, but these experiments have been inconclusive (123).

Even though these studies are so preliminary as to raise more questions than they answer, and even though the complexities of such processes as learning and memory cause one's mind to reel, the fact of their molecular origin should suggest the probability of genetic variation in their expression. What forms they would take is unclear, nor is it clear how one would ever be able to ascribe a genetical variation in human learning or memory to molecular differences, but the fact that proteins are involved does suggest the possibility of single gene variants in those functions. One form of memory defect, already alluded to, may be reading disability, a deficit in learning which has been attributed in some persons to defects in visual or auditory memory.

Language.—There is an innate capacity to develop facility in any of the world's languages or in any that can be devised. This function has been studied mainly by psycholinguists and, latterly by biologists, and the literature reflects the differences in terminology and approach.

The biologist's description of what is innate is of a species-specific capacity to employ cognitive processes to express relations and to construct a language from the raw materials available, that is, from external stimuli (124, 125). Language is not a tool created for use, but a part of the operation of the brain, and as much a part of the individual as his physical attributes. The linguists speak of a universal or innate grammar, subject to the constraints of the nervous system, and capable of being transformed to a common communication system compatible with local environments (126, 127). The brain possesses the analytical capacity to separate speech sounds from those of noise.

Evidence of the biological basis of language development is derived from several lines of investigation (124, 125). First, the rate of acquisition of facility with language is correlated with motor development and is nearly impervious to efforts to hasten it. The rate of development is largely independent of the amount or quality of the language to which the child is exposed, and while poor stimulation is not conducive to good performance, specific training programs cannot produce outstanding performance in all persons. Children learn languages from the adults in their world, but they do not learn grammar by imitation (126, 128, 129) The grammar of early childhood is very different and has syntactic rules which change with development. For example, a child cannot remember and repeat sentences of syntactic complexity incompatible with his developmental stage (128). Finally, the ease with which facility with new languages can be achieved appears to fall off in the early teen years, never to be resumed (124).

All of this suggests individual internal direction in acquisition of language and we may, therefore, expect to find genetically determined variability. For presumed genetic variation in language, the evidence in the literature is less abundant and less precise than that for reading variations. There are a few pedigrees of families containing persons said to have congenital language disability and Luchsinger has studied twin pairs (130, 131). The concordance of the MZ twins was usually considerably higher than that of the DZ pairs, suggesting some sort of genetic causes of the difficulty. There are also reports of some otherwise intelligent persons who never manage to master grammatical rules easily learned by preschool children (127). Such perversity in the face of heavy social pressures to conform to rules of speech could only be the result of some permanent CNS disorder, whether genetic or acquired.

In summary, language development and use have not been studied much from the viewpoint of individual variation. Tests now available which discover how much a person can learn about language and how he learns it have not been used to detect genetic differences, and the number, nature, and functional characteristics of the presumed genes will have to await discovery of their existence.

BEHAVIORAL VARIATIONS IN DEFINED GENOTYPES

The foregoing will have revealed the remoteness of the broad phenotypes of behavior from their genetic origin, and their resistance to an analysis which might reveal the number, the kinds, and the functions of the genes involved. A second way to study behavior genetics is to examine the effects on behavior of known genotypes.

CHROMOSOMAL ABERRATIONS

Although changes in nucleotide sequence may be the major source of genetic variations, alterations in structure and number of chromosomes are responsible for some. As with point mutation, there is a spectrum of expression beginning with lethality or severe abnormality on one side, and extending to apparent lack of manifestation on the other. The former are rare and extensively investigated, while the latter are common and neglected. This is due, no doubt, to medical exigency, but the minor variants, including anomalies of size and shape and small deletions, might be kept in mind as a possible source of differences between ostensibly normal persons, and of contributions to human variability.

Autosomal syndromes.—Human cells tolerate major abnormalities of autosomes very poorly. Most affected persons are severely malformed, so that the behavioral aspects of most of these syndromes are the product of profound distortions of development. Mosaicism, however, may enable less abnormal persons to survive, but studies of the behavioral characteristics of such patients must be very few.

Until recently it has been impossible to define precise relationships between individual chromosomes and phenotypes because no one knew which chromosome was which. Now, staining methods can characterize each chromosome in preparations of somatic cells (132). This means, for example, that we know unequivocally that mongolism is due to trisomy of chromosome 21, and so the attributes of mongolism must be presumed to be due to the effects of the presence of the extra chromosome itself, or to the third dose of each of the genes it carries. In addition, the use of deletion mapping techniques and cell hybridization promise rapid accumulation of human linkage groups which will be associated with specific chromosomes. It may become possible then to say something about the biochemical effects of the presence of only one or of three doses of specific genes in deletions or trisomies, respectively.

Since the degree of severity of the abnormality is proportional to the amount of genetic material deleted or added, mongolism is over-represented among survivors with chromosome abnormality, and a number of behavioral studies have been done on such persons. For example, Lenneberg studied 21 mongoloids over a 3-year period and showed that the stages of language development were more clearly related to chronological age and motor development than to intellectual ability as expressed in the standard I.Q. tests (133). Others have compared scores for psychological tests of mongols with those of persons with other kinds of retardation and, although results are conflicting (134, 135), one multivariate analysis distinguished mongoloids from patients with undifferentiated retardation, and both of these from patients with phenylketonuria (136).

We have no idea how an extra chromosome 21 causes the complex of developmental abnormalities we call mongolism, although all of the damage must be the result of the physical presence of the chromosome or of an excess of normal genes. Yielding has offered the interesting hypothesis that the abnormalities in this and other trisomies are due to an imbalance of regulatory and structural genes caused by the presence of the third chromosome (137). Too many regulatory genes produce too much repressor substance which prevents structural genes from fulfilling their developmental missions. This idea is derived from the observation in bacteria that structural expression is inversely proportional to the concentration of repressor. Unfortunately, there is no way to test it in mammalian cells. On the other hand, there is no doubt that there are biochemical abnormalities in the cell of mongols (138). Some of these are: a decreased rate of replication of mongoloid cells grown in vitro (139); a shortened lifespan of polymorphonuclear leukocytes (140); decreased immunoglobulins; and elevation of serum uric acid (141).

These are only a beginning of the biochemical characterizations of mongolism. Future tests should be done on patients of different ages and development and expressing the full range of behavioral variations. Correlations of chromosomal, biochemical, and developmental variation with behavioral characteristics might be enlightening.

SEX CHROMOSOMES

Anomalies of the sex chromosomes also consist of abnormal structure or number, the latter predominating, but the protective effect of inactivation of all but one X chromosome, and the relative genetical inertness of the Y, allow the viability of deletions and polysomies which, if autosomal, would be lethal. On the other hand, affected persons do not escape altogether and the general rule is that the more abnormal the karyotype, the more abnormal the phenotype; for example, average I.Q. is said to decline with increasing numbers of X and Y chromosomes (142). This declension is paralleled by other more subtle differences. For example, Penrose has shown that on the average the fingerprint ridge count is inversely proportional to the number of sex chromosomes (143). Thus, 45 XO patients have the most and the others follow in the progression XY>XX>XYY>XXX, and so on. Evidently, the Y chromosome exerts a less depressing effect on the ridge count and on I.Q. than the X.

Although some individuals with each of these syndromes show behavioral differences, the methods of ascertainment of the cases and the incompleteness of the behavioral data in many of the papers presented allow only tentative estimates of the frequency of mental retardation, psychosis, or other behavioral abnormality, and even leave us unsure whether the incidence of some of these qualities is actually greater among the chromosomally abnormal than in the general population. Since most of the studies have been done with cytogenetic aims in mind, detailed psychologic examination and even family histories are usually lacking.

1. MENTAL SUBNORMALITY

There is no evidence that 45 XO patients are more frequently mentally retarded than normal persons, but perhaps 25 percent of 47 XXY males are (142). These figures are reasonably accurate because the patients are ascertained through a variety of sources, while all that can be said of 47 XXX patients is that they appear in institutions for the mentally retarded with frequencies several times those of unselected newborn infants, and of 47 XYY, that some of the cases discovered in prisons or reported as isolated cases are retarded, usually mildly (142). Patients with four or five sex chromosomes are usually severely retarded, although some 48 XXXY patients are said to have normal intelligence (144).

Few of the reports of these patients give details of psychological tests. In general, 47 XXY patients make equal scores on the verbal and performance parts of the WISC, but there is much variation with differences in some patients of 15 to 20 points on either side, and XYY patients show the same characteristics (145). 45 XO patients are also of unusual interest. Money and his colleagues have shown that many, but not all, such persons are deficient in the WISC performance scores, do poorly in a test of direction sense, and have mild dyscalculia (146–148). Despite the handicaps in space-form perception, however, the patients do not have any reading disability (149).

2. MENTAL ILLNESS

The question of whether there are personality characteristics for each of these syndromes is even more difficult to resolve. Some 47 XXY patients are said to be immature, dependent, and passive, while others are schizoid or overtly schizophrenic, paranoid, or depressed, and appear in mental hospitals with frequencies of 3 to 4 times the incidence in the newborn population (142). 47 XXX patients also appear in such hospitals with similar relative frequencies (142, 150), and there are reports of 47 XYY patients, too, but without frequency estimates (142). But these people constitute less than 1 percent of the schizophrenics in the hospitals surveyed (142), and if the incidence of schizophrenia in the population is about 1 percent and, given population frequencies of 1/400, 1/700, and 1/1500 for 47 XXY, 47 XYY, and 47 XXX, respectively, there is some question whether their psychotic behavior has much to do with their chromosome constitution. Money queried the relationship specifically in a paper reporting one patient with 48 XXXY and one with 47 XXX, both of whom were schizophrenic and both of whom came from families containing other schizophrenic members whose chromosome complements were normal (151). These questions can be settled only by discovery of cases in some truly representative way, followed by careful medical and psychological assessment, together with some study of other members of the family. It seems likely, however, that the final story will reveal that the possession of excess sex chromosomes contributes to both mental deficiency and to personality deviation in a significant proportion of each of the syndromes and, while psychotic behavior may also be a manifestation, it remains to be determined whether such behavior is a direct result of the chromosomal aberration or whether the latter acts as a precipitating cause in genetically susceptible persons.

3. VIOLENT BEHAVIOR

The same problem is encountered in the apparent over representation of both 47 XXY and 47 XYY patients in prisons (142, 152–156). The XYY patients especially have been found in hospitals for persons confined for persistently violent and aggressive behavior, but less frequently in ordinary prisons, suggesting something particular about the compulsive and irrational quality of their behavior (155). Although the data at the moment do suggest that a person with the 47 XYY karyotype and, perhaps to a lesser degree that of 47 XXY, has a higher than average probability of spending time in prison, no one knows the magnitude of that probability. The first requirement is for surveys to discover the full spectrum of behavioral characteristics of these syndromes, after which some rational means might be designed to minimize the likelihood of the antisocial behavior of their victims (157).

The mechanisms whereby the manifestations of all of these sex chromosome abnormalities are caused is unknown. If the normal female functions with only one active X chromosome in each cell, why should the 45 XO and others with a surplus of X's or Y's be abnormal? For the polysomies, the question is whether the defects are due to an excess of certain genes or to the physical presence of the surplus chromosomes themselves (142). Inactivation of the second X is thought to occur very early in embryogenesis, so one may postulate that all of the subsequent damage is due to events occurring prior to inactivation, or that all of the genes in the inactive X's are not turned off, or that the extra chromosomes interfere in some way with replication of the cells (142).

SEX DIFFERENCES

Sex is the most obvious human dimorphism and although there is some overlap, it is infrequent. Sexual mating is a necessity for the formation of new assemblies of genes in the gametes, and is an important source of genetic variability. All species engage in sexual reproduction, although it has been achieved in different species by many different evolutionary mechanisms (158, 159). In man, the requisite number and combination of sex chromosomes sets in motion a train of events which lead to the gonadal, somatic, hormonal, social, and reproductive aspects of sex, and at each level the two sexes differ in increasingly numerous biochemical, physiological, physical, and behavioral respects (160, 161). Some of the differences are altogether cultural, but the participation of genes in the determination of such fundamental characteristics as gender role, or in hormonal effects on the brain, or in intellectual function, is receiving a great deal of attention (162, 163).

It is not yet clear whether the sex chromosomes contain specific sex-determining genes or accomplish their ends as whole chromosomes (158), but it is definite that the X, if not the Y, contains genes which are unrelated to sex. One source of sex differences resides in the possession by females of two doses of these genes in contrast to one by males. Inactivation of one X in each female cell equates the number of functional X chromosomes in all members of each sex, but since the active one in some female cells may be paternal, while that in other cells may be maternal, opportunities for gene expression are available to females which are denied to males. In addition, although the manifestations of female homozygotes and male hemizygotes may be identical, the latter will outnumber the former in the population in a ratio of q/q^2, where q equals the gene frequency. The X chromosome contains many loci, and some of these must affect behavior; for example, McKusick lists 30 X-linked neurological disorders (164). For these loci, drawn to our attention because of the mutants, and for others still undiscovered, there must be polymorphic genes as well as rare mutants whose effects are not seriously disabling and which would be expressed as behavioral differences, and the hypothesis suggests that the distribution of the variations between the sexes must be different. Nothing is known of this for any behavioral characters, but the idea is given support by observations of other kinds of characteristics in which the predicted sex difference has been found (161).

BEHAVIORAL DIFFERENCES DUE TO SINGLE GENE EFFECTS

The list of human differences due to mutants at a single locus is now nearing 2000 (164). Most of these are diseases, and many include behavioral manifestations that are often the result of the social impact of the disease and a remote effect of the gene. For example, disfiguring or incapacitating malformations or dwarfism may impair personality development or produce behavioral eccentricity. But other single gene diseases, particularly some inborn errors, have a direct impact on the central nervous system producing developmental retardation, learning disability, behavior problems, or psychotic episodes. The emphasis in the study of the inborn errors has been on their biochemical properties, perhaps because biochemical elucidation suggests therapy, while their behavioral aspects are neglected. Often it is simply stated that the patient is retarded and no details are given of sibs or of parents. In addition, although something is known about the range of behavioral abnormality for phenylketonuria, a relatively common disease, for some of the very rare ones one cannot even be sure that mental retardation may not be coincidence, the result of a bias of ascertainment.

Despite these shortcomings, some interesting general properties of these diseases are coming to light. As might be expected, all of the disorders do not alter intellectual function and behavior in the same ways nor to the same degree (165, 166). Although most investigators seem satisfied to sum up behavioral impairment as "mental retardation," tests of cognitive and other functions reveal that some of the diseases are as distinctive in the behavioral sense as in the biochemical. For example, the multivariate analysis of tests of the Guilford battery which distinguished patients with PKU, mongolism, and undifferentiated mental retardation has already been mentioned (136). Unfortunately, for most of the inborn errors psychologic testing is seldom done, although Anderson and his colleagues have reported such studies for phenylketonuric patients (167–169). Furthermore, some inborn errors may improve intellectual status, since in a small series Money has shown that the I.Q. of patients with the adrenogenital syndrome is, on the average, greater than normal (170).

There are upward of 50 biochemically defined disorders which may cause mental retardation, most of which are so rare that nothing is known about clinical or genetic heterogeneity. Among reported examples of the more common ones, however, results of psychological tests for some patients qualify in every way as normal, while others are simply said to be normal but no psychological data are given. In some instances the intellectual and biochemical variation is greater between than within families suggesting different genes, but in others, defective and "normal" sibs are reported in one family. These variations are well exemplified in phenylketonuria in which at least two, and possibly more, biochemically and genetically distinct syndromes have been described, each different in chemical attributes and in the incidence of mental and behavioral abnormality (171, 172). Persons with normal, even above average I.Q., appear in each of these classes, and some of these patients exhibit delayed speech development, behavior or emotional problems, or even psychotic manifestations (173–175). To what extent these expressions are the result of selective injury to the developing brain or of continual irritation by abnormal metabolites, or both, is not clear. The fact that hyperactivity and irritability are diminished by treatment of older children whose brain development is already irreparably impaired suggests that at least some of the behavioral abnormality is due to circulating metabolites. Whether or not some patients whose intellectual endowment has been preserved by treatment will suffer such effects will be seen as they go off the protective diet (176).

Phenylketonuria serves as a model for other, less frequent, disorders predicting clinical, biochemical, and genetic heterogeneity in them as well. Table 3 gives a list of diseases in which some cases have been reported to show behavioral expressions other than retardation. An important question posed by these data is whether these inborn errors produce psychotic or other behavioral disorders in persons who are genetically susceptible or are themselves causes of the behavior. The question could be answered by discovering whether those who exhibit psychotic behavior, for example, have relatives who are also psychotic, while those who do not show such behavioral aber-

TABLE 3. Variations in Behavior Other than Severe Mental Retardation in Patients with Inborn Errors of Metabolism

Disease	Mental Development				Defects in speech development	Psychotic (+) or abnormal (*) behavior	References
	Normal	Usually, but not always normal	Usually, but not always retarded	Retarded			
PKU			+		+	+	173–175
Histidinemia		+			+		181, 182
Homocystinuria			+			+	182
Cystathoninuria			+		+	+	183–185
Aspartylglucosaminuria				+		+	186, 187
Hartnup disease		+				+	188
Porphyria	+					+	189
Wilson's disease	+					+	190
Cystinuria	+					+	191
Lesch-Nyhan disease				+(?)		*	192, 193

ration have only normal relatives. The list is short, but it is predictable that other diseases will be added when new ones are discovered, or when the full extent of heterogeneity of known ones is apparent.

The probability of discovery of many more inborn errors is very high. Garrod himself expressed his belief that the variations he studied represented only the visible portion of a very large spectrum, and genetic principles predict that there should be an inborn error for every gene. If so, and if persons with known enzyme deficiencies show delay in speech development or learning disorder, it is quite possible that some persons with, for example, reading disability, may have other inborn errors as yet undescribed. The question is, who is a likely prospect and what screening procedures should be applied. The former question is perhaps easier to answer than the latter. Learning disability clinics of all kinds might be good hunting grounds, in the way institutions for the retarded have been in the past, but the invention of screening techniques to discover new metabolic variations is not yet far along.

If homozygous conditions lead to severe mental retardation, might not heterozygous states be expressed in some persons in some mild form, perhaps in some selective brain dysfunction? The evidence for PKU suggests that there might be a higher incidence of psychotic and other mental abnormality among parents and other relatives (177–180). For example, in two studies, plasma phenylalanine levels of patients in mental hospitals were significantly higher than those of normal persons, but not as high as those of known heterozygotes (178). These are leads to pursue; tests of reading and of other cognitive functions should be done. Variations in any significant number of these persons would be widely reflected since the frequency of PKU heterozygotes in the United States must be of the order of 1 or 2 percent. Not much is known of the effects of other genes in heterozygotes and not much has been looked for. If the average incidence of each of 50 recessive inborn errors causing mental retardation is one per 50,000 persons, then nearly one half of the population would be heterozygous for one or other of these genes. Even if the average incidence were only one per 100,000 persons, the incidence of heterozygotes is still about 30 percent of the population. Although many of these mutants are likely to be harmful to no one, and none is likely to be harmful to everyone, some are likely to exert an effect in some persons which would be in the general direction of that seen in the homozygotes. It is at least an hypothesis to act upon by making careful observations of intellectual and behavioral attributes of the parents and sibs of patients with inborn errors.

The cumulative effects of such genes in the whole population may be very great. If it is true that on the average we all have several genes which, in homozygotes would be lethal and if, as is predictable, there are many more inborn errors than we now know, it is plausible to suppose that we might each have some behavioral peculiarities which are due to the possession of rare mutant genes.

CONCLUSION

The great virtue of the genetic method is its predictive quality. Given some facts about the nature of the genetic material, its replicative capacities, its relationship to cell metabolism, and the mechanisms of its transmission across generations, one can construct a set of rules of biology whose validity has been supported repeatedly by empirical observation. Therefore, we must expect genetic inequalities among human beings in all attributes, including behavior, and that the genetic component of complex behavioral phenotypes will be very heterogeneous. Furthermore, the genetic analysis can be used to detect and describe the components of phenotypes which are the product of the action of many genes in many environments. The method is maximally efficient when used in the fashion of Benzer and his colleagues in an organism which is easily manipulated and whose mutation rate can be arbitrarily enhanced, but in modified form similar explorations can and should be carried out in human systems too. No doubt we can never uncover the molecular basis of genetic variations originating in embryonic life or which are the result of subtle anatomic differences in the central nervous system, but the clinical, physiological, and biochemical attributes defining many behavioral phenotypes await description and characterization.

The value of knowing the genetics both of abnormal and normal behavioral phenotypes is self-evident. For the former, such knowledge may lead to rational treatment, for the latter it may lead to recognition of gifts to be exploited and weaknesses to be strengthened if possible or circumvented if not. Modern society places heavy educational and technical demands upon its citizens, many of which fall equally upon all without regard for individual qualifications to meet them. The psychological and social penalities for failure to meet such demands are at the least disheartening and are sometimes disastrous, and too little attention has been paid by psychiatrists and sociologists to possible genetic causes of such inadequacies (194). The first step is to acknowledge the role of the genes in behavior and, while no one expects dramatic progress to begin with, knowledge of the genes and how they work will move us one step toward a more rational society.

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LEARNED HELPLESSNESS

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Experimental psychologists interested in learning have traditionally studied the behavior of animals and men faced with rewards and punishments that the subject could control. So, in a typical instrumental learning experiment, the subject can either make some response or refrain from making it and thereby influence the events around him. Nature, however, is not always so benign in its arrangement of the contingencies. Not only do we face events that we can control by our actions, but we also face many events about which we can do nothing at all. Such uncontrollable events can significantly debilitate organisms: they produce passivity in the face of trauma, inability to learn that responding is effective, and emotional stress in animals, and possibly depression in man. This review is concerned with the behavioral and psychological impact of uncontrollable traumatic events.

Behavioral manifestations.—When an experimentally naive dog receives escape-avoidance training in a shuttle box, the following behavior typically occurs: at the onset of the first painful electric shock, the dog runs frantically about, defecating, urinating, and howling, until it accidentally scrambles over the barrier and so escapes the shock. On the next trial, the dog, running and howling, crosses the barrier more quickly than on the preceding trail. This pattern continues until the dog learns to avoid shock altogether. We have found a striking difference between this pattern of behavior and that exhibited by dogs first given uncontrollable electric shocks in a Pavlovian hammock (1, 2). Such a dog's first reactions to shock in the shuttle box are much the same as those of a naive dog. However, in dramatic contrast to a naive dog, a typical dog which has experienced uncontrollable shocks before avoidance training soon stops running and howling and sits or lies, quietly whining, until shock terminates. The dog does not cross the barrier and escape from shock. Rather, it seems to give up and passively accepts the shock. On succeeding trials, the dog continues to fail to make escape movements and takes as much shock as the experimenter chooses to give.

There is another peculiar characteristic of the behavior of dogs which have first experienced inescapable shock. Such dogs occasionally jump the barrier early in training and escape, but then revert to taking the shock;

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they fail to learn that barrier-jumping produces shock-termination. In naive dogs, however, a successful escape response is a reliable predictor of future, short-latency escape responses.

The escape-avoidance behavior of over 150 dogs which had received prior inescapable shocks has been studied. Two-thirds of these dogs do not escape; the other third escape and avoid in normal fashion. It is obvious that failure to escape is highly maladaptive since it means that the dog takes 50 seconds of severe, pulsating shock on each trial. In contrast, only 6 percent of experimentally naive dogs fail to escape in the shuttle box. So, any given dog either fails to escape on almost every trial or learns normally.

We use the term "learned helplessness" to describe the interference with adaptive responding produced by inescapable shock and also as a shorthand to describe the process which we believe underlies the behavior (see *Etiology*). The phenomenon seems widespread, and such interference has been reported in dogs by a number of investigators (3–8). Nor is it restricted to dogs: deficits in instrumental responding after experience with uncontrollable shock has been shown in rats (9–20), cats (21), fish (22–24), mice (25), and men (26–27).

Inability to control trauma not only disrupts shock escape in a variety of species, but also interferes with a range of adaptive behaviors. Rats that receive inescapable shocks initiate less pain-elicited aggression toward other rats (28), are slower to learn to swim out of a water maze (29) as are mice (25), and are poorer at food-getting behavior in adulthood when very hungry (17). Situations involving uncontrollable events other than shock can produce effects which may be related to failure to escape shock: Escape deficits can be produced by inescapable tumbling (30), passivity following defeat in fighting (31), "sudden death" following defeat (32) or restraint (33), and retardation in learning to bar press for food following uncontrollable food (34). In addition to impairing voluntary responding, uncontrollable shock produces more stress than controllable shock as measured by behavioral suppression (37–39), by defecation and conditioned fear (35), and by subjective report (40). Finally, more weight loss, anorexia, and whole brain norepinephrine depletion is found in rats experiencing uncontrollable as opposed to controllable shock (35, 36, 41).

In summary, experience with uncontrollable trauma typically has three basic effects: (*a*) animals become passive in the face of trauma, i.e., they are slower to initiate responses to alleviate trauma and may not respond at all; (*b*) animals are retarded at learning that their responses control trauma, i.e., if the animal makes a response which produces relief, he may have trouble "catching-on" to the response-relief contingency; and (*c*) animals show more stress when faced with trauma they cannot control than with equivalent controllable trauma. This maladaptive behavior appears in a variety of species including man, and over a range of tasks which require voluntary responding.

Etiology.—It is commonly accepted that animals can learn that responding controls rewards and punishments. We believe that animals are also sensitive to the independence of responding and reinforcers, and can learn that events are uncontrollable as well as controllable. The three main effects of uncontrollable trauma are explained by postulating such a form of learning:

1. Response initiation. The probability that the subject will initiate responses to escape is lowered because part of the incentive for making such responses is the expectation that they will bring relief. If the subject has previously learned that its responses have no effect on trauma, this contravenes such an expectation.
2. Retardation of learning. Learning that responding and shock are independent makes it more difficult to learn that responding does produce relief, when the subject makes a response which actually terminates shock. In general, if one has acquired a "cognitive set" in which A's are irrelevant to B's, it will be harder for one to learn that A's produce B's when they do. By the helplessness hypothesis, this mechanism is responsible for the difficulty that helpless dogs have in learning that responding produces relief, even after they respond and successfully turn off shock.
3. Emotional stress. Learning that trauma is uncontrollable may produce more stress than learning that it is controllable.

Thus, we have hypothesized that it is not shock per se but only uncontrollable shock that produces failure to escape (42, 43).

We have tested and confirmed this hypothesis in several ways. We began by ruling out alternative hypotheses: It is unlikely that our dogs have either become adapted (and therefore not motivated enough to escape shock) or sensitized (and therefore too disorganized to escape shock) by pretreatment with shock; for making the shock very intense or very mild in the shuttle box does not attenuate the phenomenon. Further, it is unlikely that the dogs have learned by explicit or superstitious reinforcement or punishment during inescapable shock, a motor response pattern which competes with barrier jumping in the shuttle box; for interference occurs even if the dogs are paralyzed by curare and can make no overt motor responses during shock (42, 43). Two direct tests of the hypothesis have been performed. If the dog has either an active panel pressing response (2) during shock in the hammock or even the passive response of standing still (7) as instrumental acts which turn off shock, he is later not helpless in the shuttle box. So, one cause of learned helplessness seems to be learning that reinforcers cannot be controlled.

Cure.—Only one treatment seems to cure helplessness in dogs. By our hypothesis, the dog does not try to escape because he expects that no instrumental response will produce shock termination. By forcibly exposing the dog to the fact that responding produces reinforcement this expectation should be changed. When "directive therapy" is used and helpless dogs are dragged with long leashes from side to side in the shuttle box, so that changing compartments terminates shock, all dogs eventually begin to respond on their own (5). The recovery from helplessness is complete and lasting, and this finding has been replicated with over two dozen helpless dogs.

The behavior of animals during "leash pulling" is noteworthy. At the beginning of the procedure, a good deal of force has to be exerted to pull the dog across the center of the shuttle box. Less and less force is needed as training progresses. A stage is typically reached in which a slight nudge of the leash will drive the dog into action. Finally, each dog initiates his own response, and thereafter failure to escape is very rare. The initial problem seems to be one of "getting going."

Prevention.—Dramatic successes in medicine have come more frequently from prevention than from treatment, and I would hazard a guess that inoculation and immunization have saved many more lives than cure. Surprisingly, psychotherapy is almost exclusively limited to curative procedures, and preventative procedures rarely play an explicit role. In our studies of dogs we found that behavioral immunization provided an easy and effective means of preventing learned helplessness. Dogs that first received escapable shocks either in the shuttle box (2) or in the hammock (44) and then inescapable shock in the hammock, learn later to escape and avoid in the shuttle box, while dogs that receive yoked inescapable shock fail to escape.

Other findings support the idea that experience in controlling trauma may protect organisms from the helplessness caused by inescapable trauma. Among dogs of unknown history, helplessness is a statistical effect: Approximately two-thirds of dogs given inescapable shock become helpless, while one-third respond normally. Six percent of naive dogs are helpless in the shuttle box without any prior experience with inescapable shock. Could it be possible that those dogs which do not become helpless even after inescapable shock have had a prelaboratory history of controllable trauma while dogs who are helpless without any previous shock have experienced uncontrollable trauma before arriving at the lab? To test this, we raised dogs from weaning in individual cages in the laboratory, and these dogs have very limited experience controlling anything, relative to dogs of unknown history. Such cage-reared dogs are more susceptible to helplessness. While it took four sessions of inescapable shock to produce helplessness one week later in dogs of unknown history, two sessions of inescapable shock in the hammock were sufficient to cause helplessness in the cage-reared dogs (8).

In this regard, we should mention the dramatic findings on sudden death in wild rats (33). When wild rats are squeezed in the human hand until they stop struggling, then placed in a water tank, they drown suddenly. Unlike nonsqueezed rats which swim for 60 hours before drowning, these rats dive to the bottom and drown within 30 minutes. Sudden death is prevented by a technique which resembles our immunization procedure: If the experimenter holds the rat, then lets it go, holds it again and lets it go, sudden death does not occur. Further, if, after holding it, he puts the rat in the water, takes it out, puts it in again and rescues it again, sudden death is prevented. These procedures, like our own, may provide the rat with a sense of control over trauma and thereby immunize against sudden death caused by inescapable trauma.

Depression.—Since most of the investigations of uncontrollable trauma have used infra-humans, we can only speculate on the relationship of learned helplessness in animals to maladaptive behaviors in man. The phenomenon of human depression, particularly reactive depression, has a number of parallels to the phenomenon of learned helplessness (45). Like learned helplessness, depression is characterized by reduced response initiation as well as a "negative cognitive set"–difficulty in believing or learning that ones own responses will succeed even when they do (46–50). In a factor analytic study of the symptoms of depression, a factor including feelings of hopelessness, helplessness, and worthlessness has been characterized as the essence of depression (46). It is also of interest that the norepinephrine depletion produced by uncontrollable shock (41) parallels the catecholamine hypothesis of affective disorders (51) which holds norepinephrine depletion to be responsible for human depression. Several recent theorists have postulated that learning and believing that one is helpless and hopeless is the central psychological cause of depression (52–54) and this articulates well with the etiology of learned helplessness. Finally, successful psychotherapy in depression, like therapy for learned helplessness, may involve having the patient come to believe that he can be effective in controlling the events that are important to him, and recent evidence lends support to this view (55–58). A caveat is in order, however: since most of the evidence for depression is largely anecdotal and selected, experimental tests in man on the helplessness theory of depression are needed.

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CHEMICAL MUTAGENS

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"Today, we human beings are exposed to a great number of substances not encountered by our ancestors, to which we therefore have not specifically adapted by natural selection. Among those substances are food additives, drugs, narcotics, antibiotics, pesticides, cosmetics, contraceptives, air pollutants and water pollutants. . . . What is needed is a scientific breakthrough that would allow ready determination of mutation frequency in mammalian cells." Herman J. Muller, October, 1963. Symposium presentation at the Food and Drug Administration, Washington, D. C.

"We need to know more—far more—about what we are doing to ourselves and our planet, and to the fetuses of our unborn children and to the genetic heritage of those children by the permissive use of chemical wonders of our age. . . . I am informed that recently developed techniques are available that offer practical procedures for routinely testing for the hazards of mutation . . . and surely it would be unconscionable, inconceivable in fact—that we should wait for time to reveal harmful mutations before we try to research out and eliminate the chemical mutagens." William D. Ruckelshaus, Administrator of the Environmental Protection Agency. Address to the American Society of Toxicology, March 9, 1971.

In the intervening years between 1963 and 1971 when these statements were made, a whole new area of toxicology has come into being whose importance to the welfare of man cannot be quantitatively stated, but in all likelihood is of profound significance. This exciting area comprises disciplines from all phases of toxicology and takes into account current advances in genetics, especially molecular genetics. The breakthrough that Dr. Muller visualized has, to a great extent, been realized in a relatively short time, although admittedly not without its share of growing pains. Nongenetically oriented toxicologists, nontoxicologically oriented geneticists as well as industrial scientists, regulatory officials, and the lay public are seeking clarification as to the meaning and importance of this new field of chemically induced mutations and how they influence life processes. In this review, the following topics will be discussed:

What is a mutation?
Molecular basis for mutation.
Repair and the mutation process.

The role of nonmammalian systems in assessing potential mutagenic hazards to man.
Procedures recommended for evaluation of mutagenic agents.
Interpretation of results.
Significance of an increase in mutation rate—medical implications.
Population monitoring.
Correlation between carcinogenicity and mutagenicity.

WHAT IS A MUTATION?

A mutation can be conveniently defined as an abrupt inherited variation that is not due to recombination of existing genetic material (i.e., exchange between homologous chromosomes). This definition would include extremely localized alterations in chromosomes involving single base pairs, or alterations extending over a significant part of the chromosome which can be detected by appropriate cytological procedures. Highly localized alterations of chromosomes (point mutations) usually can be detected by suitable analysis of the phenotypic expression resulting from the DNA alteration and following a Mendelian mode of inheritance. Table 1 summarizes known hereditary alterations.

Extensive chromosomal damage will frequently lead to nonviable cells and for this reason it might be assumed to be of less importance than point mutation in assessing public health hazards of drugs and other chemicals. Although there is no established quantitative relationship between agents that induce single gene changes (point mutations) and visible chromosomal alterations, the correlation between either physical agents or chemical agents that can cause both types of alterations has been well established (1).

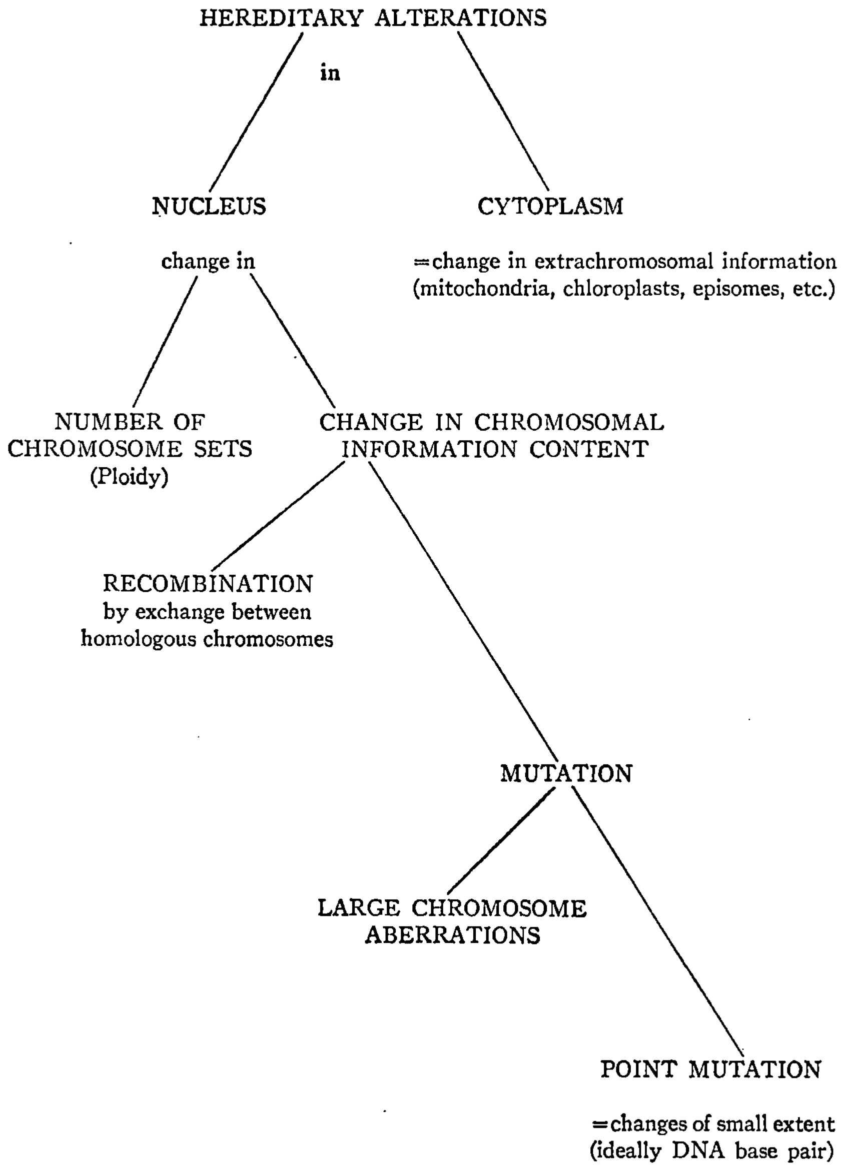
Since the correlation between agents that induce point mutations and chromosomal alterations is high, we should regard chromosomal alterations as a part of a spectrum of events produced by various agents with an additional component of the spectrum being point mutations. Figure 1 illustrates the spectrum of events induced by genetically active compounds.

MOLECULAR BASIS FOR MUTATION

In the foregoing definition of mutations, both single gene changes (microlesions) as well as chromosomal alterations are included. Mutation research, as summarized by Drake (2), is concerned with three interrelated problems: (*a*) deducing the configuration of a mutated region within the DNA molecule (i.e., the determinants of the original and mutated base pair sequence); (*b*) tracing the events by which the mutant DNA configuration arose, understanding both the primary chemical event and the fate of the "premutational lesion"; and (*c*) unraveling the complicated effects of the mutation upon the biological system.

Point mutations.—In the elucidation of the molecular basis for single gene or point mutations, microorganisms have been most useful. The haploid

TABLE 1. Subdivision of Hereditary Alterations


HEREDITARY ALTERATIONS
in
NUCLEUS
CYTOPLASM
change in
=change in extrachromosomal information (mitochondria, chloroplasts, episomes, etc.)
NUMBER OF CHROMOSOME SETS (Ploidy)
CHANGE IN CHROMOSOMAL INFORMATION CONTENT
RECOMBINATION by exchange between homologous chromosomes
MUTATION
LARGE CHROMOSOME ABERRATIONS
POINT MUTATION
=changes of small extent (ideally DNA base pair)

From Freese, E. 1970. *Chemical Mutagens*, Vol. 1, ed. A. Hollaender, 9. New York: Plenum Press. 310 pp.

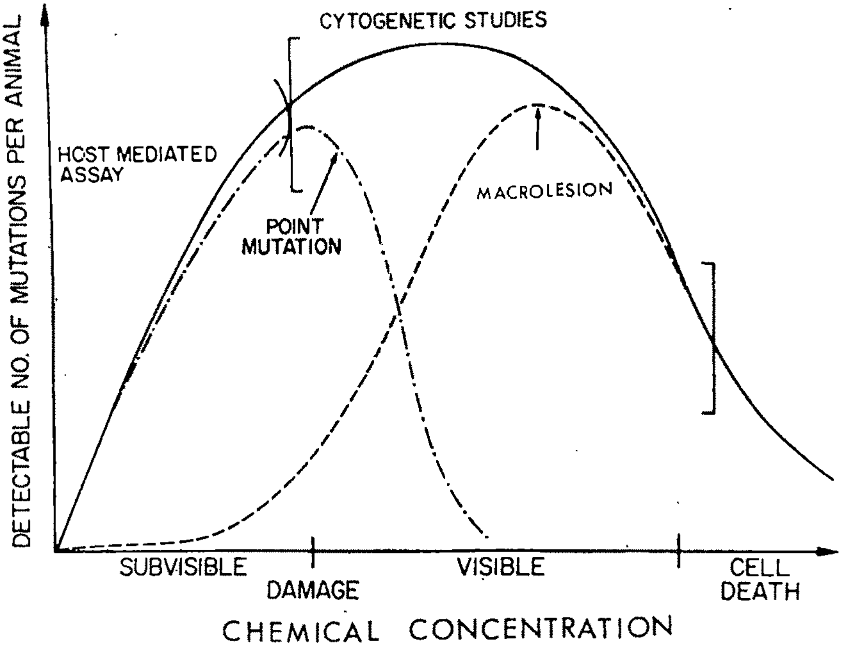


FIGURE 1. Spectrum of events induced by genetic compounds.

nature of the system and the availability of large populations have facilitated the analyses of genetic material, the site and extent of DNA alteration, and the effect on the resulting progeny. Point mutations can be divided into two main subgroups: frameshifts and base pair substitutions. Base pair substitutions can be further divided into transitions and transversions. A transition is the replacement of a purine by a different purine, or a pyrimidine by a different pyrimidine. A transversion is the replacement of a pyrimidine by a purine or a purine by a pyrimidine. Frameshift mutations consist of either deletions or additions of small numbers of base pairs (although the possibility exists for the implication of more complicated lesions). With a single base pair addition or deletion, the reading of every codon beyond the point of mutation (addition or deletion) has been changed and is read incorrectly. By adding or deleting a base, the new codon either excludes one of the bases originally present (addition) or it includes a base for a neighboring codon (deletion). Codons transcribed beyond the altered site now differ. Reading has either been shifted forward one base (deletion) or back one base (addition). The mutation will lead to the production of greatly altered proteins; see Fishbein, Flamm & Falk for detailed discussion (3).

A number of chemicals are known to cause point mutations, and their mode of action has been well established in tests utilizing microbial systems. Table 2 lists specific compounds or classes of compounds that produce point mutations. Compounds that produce base substitution cannot be reverted by chemicals that produce frameshift mutations and vice versa.

TABLE 2. Representative Mutagens and the Changes They Produce[a]

Chemical change	Mutagenic change	Representative mutagen
Base alteration—chemical change in the purine or pyrimidine bases of DNA as a result of the direct chemical action of a mutagen	Transition	Hydroxylamine Ethyl methanesulfonate N-methyl N′-nitro-N-nitrosoguanidine Nitrous acid
Base substitution—incorporation of base analogs	Transition	Bromouracil
Base removal—formation of apurinic sites as a result of chemical action	Transversion (?)	Ethyl methanesulfonate
Intercalation into DNA—insertion of materials into the DNA so that the dimensions or properties of the helix are altered	Frameshift	Proflavin ICR 170
Cross-linking of DNA strands	Deletions; frameshifts	Nitrous acid Nitrogen and sulfur mustard

[a] Strauss, B. 1970. *Chemical Mutagens*, Vol. I, ed. A. Hollaender, 147. New York: Plenum Press 310 pp.

Macrolesions.—Where large numbers of base pairs have been altered in the DNA molecule, lesions result which can often be detected by cytogenetic means. Deletions, duplications, and rearrangements are the most frequently observed chemically induced alterations. Rearrangements that are transmitted to progeny are considered the most significant cytogenetic effect, while deletions (breaks and fragments) usually produce nonviable cells. All cytogenetic alterations may be considered as part of a spectrum of events produced by genetically active compounds as previously mentioned. For a detailed review of chemicals that are cytogenetically active, see Shaw (4).

Although a great deal of information is available at the molecular level for specific agents such as alkylating agents that induce mutations, almost no information is available for the mutagenic activity of environmental agents, despite the fact that they represent many different types of chemical structures. This lack of data is most apparent with mutagenic effects of chemicals in mammalian systems. Research studies to elucidate the three major problems in mutation research are just being initiated with environmental compounds using mammalian systems. These studies with chemicals will be far more complex than that encountered with radiation, which represents a single physical agent.

REPAIR AND THE MUTATION PROCESS

The final expression of a mutational lesion in an altered phenotype is dependent on a number of factors including the type and extent of the mutational lesion and the ability or inability of the biological system to repair or alter the modified DNA segment.

Most of our information on DNA repair is derived from studies with *Escherichia coli* (5). Various kinds of damaged bases, pyrimidine dimers, certain structural defects, mismatched bases, interstrand cross-links, and single strand breaks are examples of genetic lesions that may be repaired in certain organisms.

In excision repair (one of three types of repair known in *E. coli*), the first stage, in the event of either ultraviolet or chemically induced DNA damage (i.e., cross-linkage), is physical removal from DNA of the altered bases. This is accomplished by the introduction of two single-strand breaks on either side of the lesion with the release of a single-stranded oligonucleotide containing the altered nucleotide as well as several nonaltered bases that were located on either side of the altered site. In *E. coli*, 30 nucleotide bases are released for each dimer excision. The excision by an endonuclease is limited to only one of the two strands of the DNA molecule. The gap caused by the excision process is then filled by "repair synthesis" in which the missing bases are synthesized by a DNA polymerase that utilizes the bases opposite the excised segment as a template. The final step is the resealing of the single-stranded break by a DNA ligase. The model for excision repair would call for nonscheduled DNA synthesis with nonconservative replication.

Little is known about repair mechanisms in mammalian cells but at least some of the same enzymatic steps that occur in bacteria occur in mammals. In *Xeroderma pigmentosum*, an inherited condition in humans which is characterized by an extreme sensitization of the skin to sunlight, the afflicted individual lacks or partially lacks the ability to repair ultraviolet damage to DNA. These patients readily develop fatal skin cancers from exposure to sunlight, which links lack of DNA repair with carcinogenicity (6).

An interesting application of repair in mutation research is the work of Wragg & Legator. A number of known genetically active compounds have been evaluated for their ability to induce nonscheduled DNA synthesis concurrently with an increase in DNA polymerase activity in cultured mammalian cells (7). It is anticipated that this method could identify genetically active compounds which induce repair.

It is possible that the repair mechanism both quantitatively and qualitatively may be one of the most variable processes in different biological systems. Different lines of mouse lymphoma show different sensitivity to sulfur mustards even though chemical studies show that the extent of alkylation is the same in both types of cells. Variability in repair seems to be the most likely explanation for this difference (8).

As shown in studies with *E. coli*, DNA repair prevents a potentially lethal effect; especially after an agent induces cross-linking in the genome (excision of the defective area by endonuclease). This repair mechanism which functions to enable the cell to survive does not preclude the induction of point mutations at the damaged site. The repair of lethal damage could allow nonlethal alteration to be expressed as mutations. In fact, Streissinger (9) surmised that frameshift mutations may be the consequence of mispairing errors during the repair of single-strand interruptions in a double-stranded DNA molecule.

Experimental evidence now exists that the induction of frameshift mutants in *Salmonella typhimurium* occurs as a consequence of repair (10). Repair can, in many instances, be looked upon as an attempt at cell survival which could lead to mutations. In biological systems, especially in mammals, a paucity of information exists as to the suppression or increase in mutation as a result of repair.

THE ROLE OF NONMAMMALIAN SYSTEMS IN ASSESSING POTENTIAL MUTAGENIC HAZARDS TO MAN

Almost all of the information on the molecular basis for mutagenicity and the identification of specific mutagenic agents stems from studies carried out in phage, bacteria, yeast, and *Drosophila.* The simplest organisms are usually the most suitable and yield the most significant information concerning the nature of mutation for the following reasons: 1. large population; 2. short generation time; 3. small genome (hundreds to thousands of times fewer genes in microorganisms than in man). All of the above factors facilitate the detection, classification, and characterization of mutants. The relevance of extrapolating data from such systems as bacteria and viruses to mammals is based on the premise that the basic principles of heredity, the nature of DNA in terms of chemical reactivity, stability, and the mode of protein formation are essentially the same at all levels of biological organization. Several factors, however, argue against extrapolating data from nonmammalian systems to man. Cellular uptake and distribution, metabolism, detoxification, dosage, and method of administration of the chemical are a few of the factors that profoundly affect the mutational process. It is for these reasons that little reliance can be placed on nonmammalian systems, and reliance can only be placed on mammalian species which presumably will handle chemicals in a manner similar to man. Microbial systems should be considered as ancillary procedures that can be used to answer specific questions such as the molecular nature of the lesion produced by a mutagenic agent. At the present time several examples exist of compounds that were inactive in simple systems (bacteria) but are active in a mammal; fewer examples exist for the detoxification by mammalian systems of compounds known to be mutagenic in microorganisms. While there can be little doubt that many fundamental aspects of genetics are best studied in microbes, we also recognize that higher organisms, including man, have highly complex metabolic mechanisms that are dissimilar or not even operational in systems such as bacteria. The need, therefore, to rely on mammalian systems in characterizing mutagenic agents stems not from the difference in reactivity of the genome or the various steps leading to protein synthesis, but rather from the differences in metabolism, the genetic processes dealing with repair, and possibly the fidelity of translation. One of the major reasons for the recent advances in knowledge of repair in the field of chemical mutagenesis is the availability of tests in mammals. No longer do we have to rely on nonmammalian systems to determine mutagenicity. With the development of tests in mammals, evaluating potential chemical mutagens has reached an equal status with other areas of toxicology that rely on animal studies as an indicator of potential hazard to man.

PROCEDURES RECOMMENDED FOR EVALUATING MUTAGENIC AGENTS

The value of screening compounds for mutagenic activity has been dramatically illustrated in the last few years. Without systematic screening of chemicals for mutagenic activity, and with methodologies just recently developed, these studies have already played a significant role in characterizing the genetic hazards of widely used environmental agents. The following are five examples of such studies: (*a*) characterization of the naturally occurring mycotoxin, aflatoxin, as a mutagenic agent (11); (*b*) determination of the in vivo cytogenetic effects of cyclohexylamine, a metabolite of cyclamate (12); (*c*) characterization of the widely used agricultural fungicide, captan, as a mutagenic agent (13); (*d*) determination of dominant lethal effects of the chlorinated hydrocarbon DDT (14); (*e*) determination of the mutagenic activity of N-nitroso compounds in the host-mediated assay (15).

The tests primarily used to characterize the above compounds fall into three main categories. These procedures, which can be carried out directly in animals and are currently available for conducting practical and meaningful tests, include the host-mediated assay, the dominant lethal test and the in vivo cytogenetics.

The host-mediated assay.—In this assay (16), the mammal, during treatment with a potential chemical mutagen, is injected with an indicator microorganism in which mutation frequency can be measured. After a sufficient time period, the microorganisms are withdrawn from the animal and the induction of mutants is determined. The comparison between the mutagenic action of the compound 1. on the microorganism, directly, and 2. in the host-mediated assay indicated whether (*a*) the host can detoxify the compound or (*b*) mutagenic products can be formed as a result of host metabolism. This is an extremely flexible procedure, allowing the use of a wide variety of indicator organisms and almost any suitable test animal.

The dominant lethal test.—A dominant lethal mutation is defined as a dominant genetic change that is incompatible with the survival of the conceptus. In this procedure male laboratory mice (17) are dosed orally or parenterally. The treated animals are then mated sequentially with groups of untreated females over a succeeding period of eight weeks. In mice, matings in weeks 1–3 following treatment of males represent samplings of meiotic and premeiotic stages of spermatogenesis. Females are inspected daily for vaginal plugs and are dissected in midterm of pregnancy. Females are scored for corpora lutea and for implants, comprising early deaths and living fetuses. A mutagenic index is determined relating preimplantation loss and early deaths in the treated to a nontreated control. This procedure can be conducted on virtually all laboratory animals.

In vivo cytogenetic studies.—Cytogenetic studies (18) can be carried out on almost all biological species, including man. It is the only procedure in which the results of genetic damage can be directly observed through the light microscope. In animals, somatic as well as germ cells can be analyzed, and when carried out in vivo it is a meaningful, selective assay (see discussion in the introduction). The basic requirement for conducting this procedure includes the following points: (*a*) availability of cells in active division; (*b*) accumulation of sufficient metaphase plates usually through the use of a mitotic arresting agent; (*c*) hypotonic swelling to further disperse the chromosomes within the cell; (*d*) preparation of a cellular suspension and its fixation; (*e*) slide preparation with rupture of swollen cells to spread the chromosomes in one optical plane; (*f*) photomicroscopy to obtain proper plates for analysis (19).

The above three methods can all be carried out in a wide range of experimental animals, different routes of administration can be used, and acute, subacute, and chronic studies can be conducted. The tests are comparatively simple, rapid, and economical. These procedures can be considered as relevant to man as other methods currently used in standard toxicological safety evaluation of drugs, pesticides, and other commercial chemicals. Experiences to date indicate that these three methods should be run concurrently in a comprehensive program for evaluating mutagenic agents. No one procedure can be relied upon to determine all types of genetic damage caused by various chemicals with different modes of action. The majority of mutagenic agents, however, should be detected by concurrent use of the outlined procedures.

In addition to the procedures discussed above there are a number of modifications of currently used procedures as well as several methods in the research stage that will no doubt improve our testing in the near future. A few recent developments that have yet to be investigated thoroughly are (*a*) replacement of metaphase preparation by anaphase plates in in vivo cytogenetic analysis; (*b*) use of mammalian cells in the host-mediated assay; and (*c*) scoring of translocations in meiotic preparations of mouse testes.

It is anticipated that new methods and modifications of present methods will continue to be made for an indefinite period of time. These developments should not be interpreted as a rational reason to postpone evaluating drugs for mutagenicity. The currently recommended procedures form the basis for a logical approach to evaluating chemicals, and we should continually strive to develop better methods in this area as in other areas of toxicology.

INTERPRETATION OF RESULTS

Toxicology as well as pharmacology is often criticized for not being a more quantitative science (20). Facetiously it has been said that conventional procedures for carrying out chronic toxicity studies include counting the dead, weighing everything that can be removed, slicing everything that can be sliced, and feeding everything that could be fed for two years (21). Actually, the importance of the quantitative data and the various factors that can influence a biological response in animals has long been appreciated by toxicologists and pharmacologists.

Factors influencing biological response.—The route of chemical administration, distribution, nutritional state of the animal, and especially biotransformation of chemicals entering the circulation are some of the more obvious factors contributing to the variation of biological results in the same as well as in different animal species. A given chemical at different rates may be esterified in the rat, conjugated in the dog and acetylated in man. All of these factors will influence assays for mutagenictiy as well as measurements for any other toxicological effect. Distribution of the active compound in germinal cells is obviously of key importance in evaluating potentially mutagenic agents.

Threshold concepts: similarity between carcinogenicity and mutagenicity.—The importance of a dose-response curve, the concept of a threshold effect, and difficulties of extrapolating from animal data to man, especially in the area of carcinogenicity, have been the subject of many recent reports (22). As far as can be determined the carcinogenic hazard never disappears with a diminishing dose but rather becomes infinitely small. A further complication of establishing a threshold concentration in carcinogenesis is the possibility of co-carcinogens being present in the environment, further obscuring even the possibility of obtaining a no-effect level (23).

The concept of a threshold response in mutagenesis is little different from that in carcinogenesis. In the case of X rays, a direct linear relationship between dosage and genetic effects in various biological systems gave rise initially to the "single hit" interpretation of X-ray effects (single hit on ionization of DNA). This single hit causes a permanent alteration of DNA (mutation) and the effects are usually additive when doses are applied intermittently. A number of substances (i.e., 1,2-dithioglycerol) are known to modify the lethal and/or mutagenic effects of X rays (24). The existence of compounds that can modify the mutagenic effects of X rays has cast some doubt on the target (single hit) theory and has led to the assumption that the genetic effects of radiation may be of a more indirect nature. Just as in carcinogenesis, we may have a direct effect or an effect modified by environmental agents. Since it is difficult to establish a no-effect level if, indeed, one does exist, an arbitrary safe level based on the animal data and the intended use of the chemical should be determined where possible. In the case of nonnutritional food additives an increase in mutation rate at any level may be considered inadvisable, while at the other extreme, the increase in mutation rate would not weigh heavily against the use of a life-saving drug. Between these two extremes various safety margins depending on use of the product could arbitrarily be calculated.

In mutagenic studies a dose-response curve for interpreting animal results should be a prerequisite. Mathematical models are presently under investigation that will take into account the size of the animal population studied as well as the dose-response in extrapolating to a large population exposure (25).

Precision of data.—The precision of the test used for mutagenicity should be precise enough so that a doubling of the control level of mutation would be statistically significant at the 5 percent level. A drug should be considered as a potential mutagen if one or more of the procedures are positive.

SIGNIFICANCE OF AN INCREASE IN MUTATION RATE—MEDICAL IMPLICATIONS

Environmental agents and mutation.—There can be little doubt that genetic improvement of the human species is based on gradual transformation through the occurrence of an orderly increase in the mutation rate, with new mutations conferring a selective advantage to the individuals who carry them. The mutation rate should never exceed the power of selection to remove harmful genes, for any such increase would lead to genetic degeneration. There is evidence to indicate that individual genes are extremely stable, and the occurrence of new mutations is an extremely rare event on a per locus basis, but fairly frequent when considered on a per gamete basis. In response to his environment through the ages, man has been able to perpetuate the infrequent beneficial mutation and through the process of natural selection reject the harmful mutation. It is a continual striving of the species for what has been termed the "adaptive peak."

What factors in our present environment may currently be operating to either alter the mutation rate (the production of deleterious mutations), or the forces of natural selection (removal of harmful mutations)? The advent of modern medicine, and especially antibiotic therapy, is having a profound influence on natural selection and the prevalence of genetic disease. Individuals with undesirable genetic traits are living longer and reproducing due to improved medical practices.

If the process of natural selection is less active in the removal of undesirable traits than it was 50 or even 20 years ago, what can we theorize about environmental factors that may possibly affect the mutation rate? In the last 30 years there has been a dramatic increase in the number of environmental agents to which we are all exposed—food additives, pesticides, antibiotics, drugs, and industrial chemicals. Many of these biologically active agents are recent additions to our environment and were not present 20 or 30 years ago; hence, we are besieged with chemical compounds to which we have not become adapted through the normal process of natural selection. The induction of mutations by various substances in a manner analogous to radiation has been known since the 1940's and there can be little question that among the recently introduced environmental agents many mutagenic elements are present. In the last few years, several widely used chemicals have been shown to be mutagenic in animal tests. It is conceivable that within the last few decades we have managed to neutralize the process of natural selection partially, and simultaneously to increase the mutation rate by our exposure to mutagenic agents.

Consequences of deleterious mutations.—Exposure of a random breeding population to mutagenic agents for several generations should theoretically adversely affect the population fitness by increasing the frequency of alleles with detrimental effects. The overwhelming majority of mutations either are neutral or adversely affect the species in which they occur. In humans, mutations are known to cause a variety of effects as genes influence every part of the body and control every metabolic process. Mutations can induce lethal effects which, at an early stage in development, induce embryonic lethality, miscarriages, or premature death. In addition to lethal effects, mutations are known to be responsible for physical abnormalities, and to play a major role in the causation of mental deficiency and mental diseases. From the very severe consequences of mutations at one extreme, we have mild effects at the other which may be imperceptible. The consequence of harmful mutations, therefore, ranges all the way from lethal effects to sterility, semi-sterility, and effects too slight to be detected. Inborn errors of metabolism, congenital malformation, and a host of physical and mental disabilities that affect the fitness of the population can be attributed to deleterious mutations. While lethal or sublethal mutations will be rapidly eliminated from the population, and need not be of great concern, nonlethal mutations will tend to persist through several generations with the duration of their persistence being inversely proportional to the severity of their effect. If the mutations are mainly recessive, they will be expressed only in the homozygous state, while dominant mutations will be expressed in a single generation and semidominant mutations can be expressed in either the heterozygous or homozygous condition.

Mutations as a medical problem.—In the last few years, quantitative data have become available as to the frequency of chromosome abnormalities in our population. Approximately 33 percent of all detected spontaneous abortions in the first trimester of pregnancy are chromosomally abnormal. Since 15 percent of all detectable conceptuses are aborted, this means that at least 5 percent of all conceptions carry a chromosomal abnormality (approximately 200,000 per year in the United States). There are approximately 7 either structural or numerical chromosome abnormalities detected per 1000 live births (4).

If we conclude that chromosome abnormalities are a part of a spectrum of genetic events, probably appearing after multiple gene damage, we may infer that the frequency of medically significant gene mutations is extremely high. One of the methods for evaluating the effect of gene mutations and disease is by the study of abnormal enzyme activity. There are now nearly 90 known diseases which vary from crippling to lethal which can be characterized by a marked lowering of enzyme activity probably due to the presence of abnormal proteins. Childs (26) recently calculated the number of admissions to Johns Hopkins Hospital for genetic and nongenetic causes. He concluded that one patient in 14 has a definite genetic disorder, 30 percent of the admissions may or may not be due to genetic disorders and 50–59 percent are not of genetic origin. The best approximation would be that 15–20 percent of patients admitted to the hospital had diseases in which genes played a large role. Table 3 summarizes the classification of genetic disorders used in this study. In addition to this list, the following disorders are classified as probably gene influenced: diabetes mellitus, dwarfism, hypoglycemia, hypopituitarism, obesity, pubertal variations, and thyroid diseases. Childs believes that, although many patients are admitted to the hospital with diseases due to genes at a single locus, perhaps in twice as many there is evidence of partial genetic causes but the number and kinds of genes involved are unknown.

Although the incidence of genetic disorder in our population is high, we know of only a small fraction of the actual percentage of genetic diseases in the human population. In addition to those clinical conditions which have not been characterized as being due to genetic origin, there is no doubt that a variety of subclinical conditions, which stem from deleterious mutations, contribute to the general unfitness of the race. Rough overall estimates would indicate that 5 percent of the population is mentally retarded, often due to hereditary disease; perhaps one newborn baby in 50 has a significant congenital malformation, sometimes hereditary; and some calculations indicate that as much as 2–3 percent of the population have some hereditary trait which constitutes at least a handicap and at most a life-threatening disease (27).

Lederberg (28) recently estimated that 25 percent of our health burden is of genetic origin and that the "genetic legacy" of the species will, in the near future, compete only with traumatic accidents as the major factor in health.

TABLE 3. Number and Frequency of Genetic and Nongenetic Diseases in the Pediatric Service, Johns Hopkins Hospital, July 1, 1963 to June 30, 1969[a]

Cause of Admission	Number of Cases	Frequency
Single gene defect	597	.064
Chromosome abnormality	70	.007
Total Genetic	667	.071
Possibly gene influenced	2941	.315
Nongenetic	4982	.532
Unknown	763	.082
Total	9353	1.000

[a] See reference 26.

Although we can roughly approximate the high cost of deleterious mutations on our population it is almost impossible to determine what part of our genetic burden is due to drugs, pesticides, food additives and other environmental agents. We can only theorize that chemicals probably play a major role in the induction of mutations as compared to physical agents, viruses, or so-called spontaneous mutations.

POPULATION MONITORING

In discussing population monitoring, we can define two specific problems: 1. detecting overall changes in gene frequency and 2. determining the contribution of a specific agent or agents in altering the mutation rate.

Our ability to determine changes in the mutation frequency, much less ascertain a specific cause and effect for any single mutagenic chemical in the human population, is extremely limited. The long-term nature of the process, the number of factors that can influence mutation rate and natural selection, the prevalence of genetic disease in the population and the fact that any increase in genetic effects probably would be statistical rather than unique, militates against our ability to monitor effectively the human population for mutagenic effects. Neel & Bloom recently described three possible approaches to the detection of environmental mutagens (29): 1. Comparing the children being born in two populations—one exposed to a potential mutagenic agent and the other not—with respect to a number of genetic traits. Abortion and stillbirth rates, frequency of congenital malformations, birth weight, sex ratio, early death rates, and physical development would be assessed in the two populations. 2. Measuring, in a defined population, the occurrence of certain "sentinel phenotypes." Achondroplasia, aniridia, retinoblastoma, partial albinism with deafness, microphthalmos-anaphthalmos without mental defects, Marfan's syndrome, and multiple neurofibromatosis were the phenotypes suggested for monitoring. 3. Cytogenetic and biochemical studies. These studies could be carried out on newborn populations in hospitals. In the biochemical approach, mutations characterized by changes in protein structure or function would be examined.

The biochemical and cytogenetic approach would be the most precise of the three alternatives. At present, no meaningful population monitoring now exists for detecting induced mutations, and it is highly unlikely that a program of this kind will be established in the near future. It is well to realize the shortcomings in our ability to monitor the human population, but this should in no way influence our attempt to limit the number of potential mutagenic agents in our environment.

CORRELATION BETWEEN CARCINOGENICITY AND MUTAGENICITY

The implication of a mutational event as one of the prerequisites for induction of tumors dates back to a period substantially earlier than the formation of the somatic mutation theory of cancer in 1928 (30). The heritable changes produced in the phenotype in both carcinogenesis and mutagenesis superficially made the somatic theory of cancer attractive. Many alternatives to the somatic theory of carcinogenesis can be found in the literature with one of the major theories relating to viral oncogenesis. According to this concept the chemical carcinogen activates a latent virus to induce neoplasia. This theory is not in apparent conflict with the somatic theory, as in both cases we are dealing with an altered genome.

On the basis of available information, Burdette in 1955 (31) concluded that somatic point mutations cannot be regarded as a general mechanism of cancer initiation. In the intervening years, with an understanding of the molecular basis of mutation and by the evaluation of compounds for carcinogenicity and mutagenicity in comparable systems, the relationship between mutagens and carcinogens has become clear. In a recent survey of the literature, Miller concluded that many and perhaps all carcinogens are mutagens and that many, but possibly not all, mutagens are carcinogens (32). Our ability to develop the present strong correlation between mutagenic agents and carcinogenic compounds is a direct consequence of our ability to determine both effects in mammalian systems. Prior to the development of mammalian tests for mutagenicity, mutagenic agents were evaluated primarily in bacteria or *Drosophila* and chemicals were evaluated for carcinogenicity in rodents. The major exceptions that were previously found for carcinogens not being mutagenic have been for the most part resolved by the use of rodents for testing both types of activity. Carcinogenic compounds such as cycasin and nitrosamines, which are not mutagenic when tested directly on bacteria, are now known to be mutagenic as well as carcinogenic when the active metabolites are evaluated (16). Procedures such as the host-mediated assay have contributed greatly to the establishment of a qualitative correlation between carcinogenic and mutagenic compounds. The use of available mammalian tests for mutagenicity should identify carcinogenic compounds in a fraction of the time needed for the conventional carcinogenic assay.

CONCLUSION

Within a few short years a new area of toxicology has developed, and this development has occurred without the emotional impetus of a national disaster such as the "thalidomide affair." Three major factors are responsible for the orderly development of this field: (*a*) the realization of the increasing predominance of genetic diseases as a prime factor in the health of our population; (*b*) the supposition that drugs and other environmental agents induce detrimental mutations; and (*c*) our ability to evaluate chemicals for mutagenic action in animal tests in a manner quite analogous to testing agents for other types of toxicity prior to human exposure. It is quite conceivable that evaluating chemicals for mutagenicity and their eventual elimination or curtailment will be of greater significance to the well-being of the human race than the elimination of chemicals for any other known toxic effect. The induction of an increase in mutation rate is unique in that it adversely affects not only the contemporary generations but, most importantly, future generations. It is encouraging to know that we are now testing many drugs and other chemicals for mutagenic effects by available practical methods, and we can with confidence predict that in the near future all drugs and chemicals will be evaluated for mutagenicity by constantly improving methods.

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EFFECTS OF ORAL CONTRACEPTIVE AGENTS AND SEX STEROIDS ON CARBOHYDRATE METABOLISM[1]

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INTRODUCTION

It is not the purpose of this review to describe in detail the results of several individual investigations concerned with the influence of oral contraceptive agents on glucose tolerance. This has been done previously (1–3). Instead, a general interpretation of the work to date is offered. Similarities and differences among various synthetic sex hormones are emphasized, and comparisons of these data to those pertaining to sex steroids not employed in oral contraception are made.

CHEMICAL STRUCTURE OF ORAL CONTRACEPTIVE AGENTS

Estrogenic components of combined or sequential agents are synthetic modifications of estradiol and take the form of mestranol (3-methoxy-ethinylestradiol), or, less commonly, ethinylestradiol itself. The progestin ingredients are more closely related to 19-nortestosterone (norethindrone, norethynodrel, norethisterone, etc.) or to esterified 17α-hydroxyprogesterone (e.g., acetates of chlormadinone, megestrol, or medroxyprogesterone). The addition of ethinyl or ester groups at position 17 renders these steroids more resistant to hepatic degradation after initial intestinal absorption and more suitable for oral administration.

Unlike progesterone, the 19-nortestosterone compounds possess anabolic and inherent activity and their propensity for conversion to hormones like ethinylestradiol in vivo (4). Absent estrogenic action in steroids of the 17α-hydroxyprogesterone type is due to the presence of C-19 methylation and the absence of ethinyl groups at position 17 (5). On the basis of this information, it is not appropriate to equate all of their metabolic effects with natural estrogens, testosterone, or progesterone indiscriminately.

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DIABETES MELLITUS AND THE "PILL"

Initial studies of women using oral contraceptive agents revealed an incidence of diabetes mellitus ranging from 15 to 40 percent (6–8), which is considerably above the accepted figure of approximately 2–4 percent for a general population. Criticisms of this work included the lack of control evaluations before the regimen was begun and the inclusion of many women with positive family histories of diabetes.

Prospective studies of "normal" women.—The majority of prospective studies do not support earlier findings. In eleven separate investigations of over 400 women with normal glucose tolerance before treatment, an oral contraceptive regimen for periods of one month to four years did not provoke a single instance of diabetes (9–19). In six similar studies of more than 300 women, contraceptive treatment induced chemical diabetes in 4 to 12 percent (20–25).

Both positive and negative reports indicate that there is still a problem with patient selection. It is known that standard oral or intravenous glucose tolerance tests do not distinguish nondiabetic from subclinical diabetic patients, because the latter deteriorate only during periods of metabolic stress such as during pregnancy (26) or following provocation with glucocorticoids or human placental lactogen (27). Abnormal obstetrical and family histories do not delineate these patients very well, since this institution has shown no accurate correlation between these criteria and the diagnosis of subclinical diabetes with steroid glucose tolerance tests in young adult women (28).

It is possible, then, that "normal" women developing diabetes while on this regimen are, in reality, subclinical diabetic subjects who were unrecognized by standard glucose tolerance tests before treatment and who could not be properly classified by medical history alone.

Acquired subclinical diabetes mellitus among nondiabetic women on oral contraceptive agents.—While it may be argued that truly nondiabetic women do not develop overt impairment of carbohydrate tolerance while taking these agents, glucose curves, though normal, are frequently higher than pretreatment values. This is frequently accompanied by concomitant elevations of the plasma insulin response (25). Moreover, when steroid glucose tolerance tests are performed, diabetic curves result in 45 to 85 percent of the cases (29, 30). In these instances, the plasma insulin response is subnormal as in subclinical diabetic subjects not receiving contraceptive hormones (31).

Following discontinuation of the oral contraceptive regimen, glucose and insulin profiles during standard tests fall to pretreatment levels (25), and steroid glucose tolerance becomes normal again in association with a higher, more appropriate plasma insulin response (31). These results suggest that oral contraceptive agents induce an acquired form of subclinical diabetes mellitus in nondiabetic subjects that is reversible.

Prospective studies of diabetic women.—Subclinical diabetic women who develop diabetes during pregnancy and revert to normal tolerance postpartum subsequently have been evaluated before and during treatment with oral contraceptives. In three studies involving 22 women, 12 of them (55 percent) developed overt diabetes during the course of treatment (32–34). One author reported that the plasma insulin response was defective when adverse effects were observed (32). The deleterious outcome was not always reversed when treatment was stopped.

In patients with pre-existing, maturity-onset diabetes, carbohydrate tolerance frequently worsens (35), and, in Spellacy's review (3), instances of difficulty controlling diabetic women on the "pill" were cited. The paradoxical improvement of some diabetic subjects with contraceptive agents needs further documentation (36).

It appears that oral contraceptives in their present form do create diabetogenic stress. Whether prolonged administration will jeopardize carbohydrate metabolism regardless of diabetes susceptibility is not known. Two cross-sectional studies of women who received antifertility hormones for more than five years are in total disagreement on this point (37, 38).

ACTIONS OF SPECIFIC SEX HORMONES ON CARBOHYDRATE METABOLISM

Estradiol, estriol, and estrone.—Parenteral administration of one or more of these steroids has been shown to reduce hyperglycemia or insulin requirements in six different investigations of human diabetic subjects (39–44), and to improve carbohydrate tolerance in nondiabetic women (45). In two studies in which no effect was observed, the dosage was relatively low or the patients were elderly and psychotic (46, 47). The beneficial influence of these hormones on other mammalian species that are normal or diabetic has been reviewed elsewhere (48), and confirmed recently in the female monkey, rabbit, and rat (49–51).

In liver, natural estrogens have abundant effects on several metabolic pathways (52). Hexose monophosphate shunt activity and involved enzyme systems are enhanced (53, 54), glycogen deposition is increased (55), and glucagon-induced glycogenolysis is inhibited (56). More recently, this laboratory has observed a marked suppression of hepatic gluconeogenesis with estradiol in the rat (57), and others have reported that cortisol-induced increases of alanine transaminase activity are inhibited by this hormone (58).

Similar actions on glycolysis and glycogen formation have been described in the uterus (59–63). In addition, the increased penetration of glucose and amino acids into uterine muscle which quickly follows administration of natural estrogens has been linked to increased transport by an inducible carrier-mediated system (64–66). In rat skeletal muscle and adipose tissue, a number of estrogens increases the sensitivity of these tissues to insulin with respect to glucose utilization, glycogen formation, and lipid synthesis (53, 67, 68).

These results suggest that certain estrogens are antidiabetogenic through their suppression of hepatic glucose formation and augmentation of peripheral glucose utilization. It has been proposed that many of these metabolic changes are mediated by direct trophic actions on pancreatic islets (48). Hypertrophy of pancreatic islets, increased islet insulin secretion, and hyperinsulinemia do follow estrogen administration (48, 50, 69). However, progesterone has similar properties without much benefit on glucose tolerance (50, 69–72). Moreover, natural estrogens improve diabetes in humans on fixed doses of insulin. It is concluded that although some sex steroids may have a direct influence on endocrine pancreas, there must also be some additive effects or synergism between insulin and estrogens to explain the foregoing observations.

Progesterone and 17α-hydroxyprogesterone.—Progesterone duplicates the effects of natural estrogens with respect to increased pancreatic islet insulin secretion and plasma insulin dynamics, but has little influence on carbohydrate or tolbutamide tolerance in normal monkeys and man or on the course of experimental diabetes (48, 50, 70–72). Progesterone does not share many of the actions of these estrogens on carbohydrate metabolism at organ sites with the exception of certain pathways in liver (57, 73).

Although this steroid blunts the hypoglycemic response to insulin, suggesting insulin antagonism, it reverses the hyperglycemic effects of glucocorticoids (70), suppresses plasma growth hormone (74), and promotes adipose tissue deposition (75). When given in combination with estrogens, it does not counteract their beneficial effects (44, 50). Thus, the evidence for a major contra-insulin action of progesterone is not striking, but more studies of human diabetic patients are needed to support this conclusion. Unfortunately, similar investigations with 17α-hydroxyprogesterone have resulted in conflicting reports of both beneficial and antagonistic effects on glucose tolerance (76, 77).

Synthetic progestins.—No significant alterations of carbohydrate tolerance have been reported in normal or diabetic patients following the administration of these compounds alone in amounts comparable to the "minipill" or conventional oral agents, and plasma insulin changes usually are minor if they occur (14, 78). With higher pharmacologic regimens in Rhesus monkeys, these progestins usually boost the plasma insulin response but, in contrast to the 19-nortestosterone compounds, those steroids related to 17α-hydroxyprogesterone improve carbohydrate tolerance (2, 70, 79).

Two of these hormones, megestrol and medroxyprogesterone, have methyl substitutions at position 6. This configuration greatly enhances steroid-induced hepatic glycogen formation (80), which may be indirect evidence for reduced glucose output by liver and explain improved glucose tolerance. Chlormadinone, with its 6-chloro substitution, has similar effects on normal and diabetic animals (79, 81), but depot forms of long-acting medroxyprogesterone may worsen human diabetes (82).

Other natural and synthetic estrogens.—Conjugated equine estrogens such as equilenine or Premarin® have no beneficial action in animals (48) and may provoke mild-to-moderate deterioration of carbohydrate tolerance in some human subjects (83). Similar results may attend the use of oral diethylstilbesterol, ethinylestradiol, and mestranol in humans (21, 29, 30, 83–85), which is reminiscent of reports of glucose intolerance in force-fed normal and diabetic rats after short-term administration of similar estrogens (86). However, Houssay and others maintain that following an initial diabetógenic effect of estrogens like stilbesterol, carbohydrate metabolism eventually improves in the diabetic rat (48). This observation also deserves further confirmation in human diabetic patients.

Although oral estrogens alone can mimic the diabetogenic effects of oral contraceptive agents, one cannot discount the possibility that some progestins intensify this action by undefined mechanisms or through their own estrogenicity, whereas other progestins do not. Spellacy's data ostensibly support this conclusion when effects of mestranol plus ethynodiol are compared to mestranol plus chlormadinone in two groups of women (11, 24).

There is no conclusive proof at this stage of investigation that mestranol is more diabetogenic than other oral forms of estrogens. There does appear to be a decided contrast between parenterally administered natural estrogens and orally administered synthetic and semisynthetic derivatives in human subjects. It is not known if this is attributable to different routes of administration or to basic differences in chemical structure.

DIABETOGENIC FACTORS RELATED TO ORAL CONTRACEPTIVES

Growth hormone.—Both estrogens and combined or sequential oral contraceptive agents enhance plasma growth hormone responses to insulin-induced hypoglycemia and arginine infusion (87–90). Growth hormone is a potent contra-insulin principle (91), and a disturbance of this type may explain the adverse effects on carbohydrate metabolism that these oral agents sometimes have.

However, the final outcome of estrogen administration may be beneficial or antagonistic for glucose tolerance, depending on the hormone employed, and this occurs in both intact and hypophysectomized animals (48), in human subjects with and without pituitary function (21, 29, 30, 83–85, 92), and evanescently in patients with acromegaly without significant change in plasma growth hormone (93). Also, progesterone and medroxyprogesterone administrations suppress plasma growth hormone (74, 94, 95) while exerting different effects on glucose tolerance (70–72, 79, 95). These data suggest that sex steroid influence on carbohydrate metabolism bears less relationship to changes in plasma somatotropin and more to steroid structure itself.

Adrenal glucocorticoids.—A variety of estrogens, oral contraceptive agents, and some progestins increase transcortin-bound and unbound plasma cortisol or corticosterone (96–100). Estrogens also partially inhibit hepatic reduction of ring A of the cortisol nucleus (101, 102), which contributes to the slower plasma disappearance of injected cortisol in estrogen-treated subjects (103). The intensification of the diabetogenic stress of glucocorticoids by concomitant administration of estrogens is further evidence of the positive interplay between these two hormones (104), and has obvious relevance to the very high incidence of abnormal steroid glucose tolerance tests among users of conventional oral contraceptives (29, 30). Estrogen and oral contraceptive agents elevate serum pyruvate concentrations in a manner that resembles glucocorticoid action (25), which also indicates that the elevated plasma-free cortisol concentration induced by these agents has physiologic significance.

Other factors.—There is evidence that estrogens affect rates of intestinal glucose absorption (105, 106) and gastric emptying (107), and enhance intestinal metabolic activity (108). This may implicate oral contraceptives in the higher incidence of altered oral, as opposed to intravenous, glucose tolerance tests (84). Further studies are indicated to test this possibility and to evaluate the influence of sex steroids on enteric hormones generally. Whether catecholamines or pancreatic alpha cell glucagon are modified by sex steroids and indirectly influence carbohydrate metabolism is not known.

CONCLUDING REMARKS

Effects of sex hormones on carbohydrate metabolism very likely involve their interactions with insulin (antidiabetogenic) and endogenous glucocorticoids (diabetogenic). The responsiveness of liver to all three principles in this triad, as well as the fundamental role of liver in the metabolism of steroids and control of glucose homeostasis, may place primary events in this organ. One cannot ignore similar interactions in peripheral tissues, such as insulin-sensitive adipocytes and muscle, or the role of less well-defined modifying factors including other contra-insulin hormones.

Most sex steroids augment plasma insulin at some dosage level, but the magnitude of the change in no way accurately predicts what happens to glucose tolerance. For this reason, it is not justifiable to regard all estrogens and progestins as insulin antagonists until it is determined how directly these hormones act on the beta cell in a positive trophic fashion.

Certain parenterally administered estrogens and progestins induce hyperinsulinemia and improve carbohydrate tolerance, suggesting that sex steroid-insulin interactions predominate. Whether this benefits diabetic as well as normal human subjects in all instances requires further study. Menopausal status may prove to be a critical determinant.

Conventional oral contraceptive agents and some related oral compounds appear to enhance the biologic expression of glucocorticoid activity to a greater extent. These particular sex hormones also may have intrinsic diabetogenic action of their own with respect to beta cell performance and tissue responsiveness to insulin. The degree to which adverse effects are resisted and carbohydrate tolerance is preserved appear to depend, in part, on the magnitude of plasma insulin responses. Thus, subclinical and overtly diabetic subjects, with compromised endocrine pancreatic reserve on a functional or absolute basis, are more sensitive to these regimens, and their use in such patients is relatively contra-indicated.

Future investigations in this area must ascertain more precisely the importance of hormone dosage, duration and route of administration, structure-activity relationships, and species specificity. Eventually, an ideal oral contraceptive may emerge that is satisfactory for use even in diabetic subjects.

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LITHIUM SALTS IN THE MANAGEMENT OF THE MANIC-DEPRESSIVE SYNDROME

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Lithium has had a checkered, although unique history since its discovery in 1818. Arfwedson (1) demonstrated that the mysterious element resembled sodium and potassium in some reactions but not in others. Lithium salts were later detected in the spa waters in Europe and, thus, about 100 years ago, it was first launched on its medical and therapeutic career. During the latter half of the 19th century and the early part of this century, claims were made for the therapeutic value of lithium salts in a vast array of disorders such as hypertension, gout, rheumatic gout, urinary calculi, and epilepsy. It was also reported to have diuretic, tonic, and sedative properties. None of these applications became established and most are now considered ineffective. Its next appearance was in the late 1940s in the United States, when lithium chloride became a popular salt substitute for patients on sodium-free diets. It was being taken by patients with heart and kidney disease and some fatalities and serious poisoning resulted (Corcoran, 2).

This background would not appear to be an auspicious one for any new therapeutic claims for lithium. However, in 1949 a report by Cade (3) stated that lithium salts given to 10 manic patients resulted in improvement in the conditions of all the cases. This report includes the trial of lithium in six patients suffering from dementia praecox with no fundamental improvement noted in any of them, except some decrease in restlessness in three. The medication was also tried in three patients suffering from chronic depressive psychoses, again without any improvement. Thus, it is these uncontrolled observations by a clinician that have been explored over the past 20 years.

ACUTE MANIA

There are to date over 70 published reports on the use of lithium for the treatment of mania. With only one exception, all report on the clinical effectiveness of lithium in acute mania and hypomania. In a recent review of the literature, Gershon (4) reports that improvement rates vary from 60 to 100 percent. Other surveys, (Tupin, 5), give 78 percent improvement in over 1100 cases while Schou (6) places the average improvement rate at 70 to 80 percent. From these data, one might conclude that lithium is an effective agent for the treatment of mania. Unfortunately, in most studies, the lack of a control group makes it difficult to evaluate critically the real efficacy of lithium in these reports. The literature includes trials in manic patients, schizophrenic patients, and those with mixed diagnoses. Methodological problems are perhaps greater in the study of mania than in any other psychiatric condition, but additional difficulties arise from special problems in differential diagnosis, in the self-limiting and recurrent nature of the disease. These methodological questions are of considerable importance in evaluating published reports in this area and are discussed in more detail elsewhere (Gershon, 7). In most of the studies in the literature, no rating scales were employed and often either concomitant medications were employed or there was a lack of an adequate drug washout period. Thus, since no rating scales were employed, the term "improvement" may vary greatly in significance. Also, the large variation in reports of the incidence of the disease tends to suggest a large variation in diagnostic criteria, which could account for marked differences in the treatment outcome in different studies.

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With these reservations, a review of the available controlled studies will be presented. Nine such studies exist in the world literature. Lithium has been compared with placebo (4 studies) and with chlorpromazine (5 studies). It is relevant that there are no other control studies in mania for any other therapeutic agents. All four placebo-controlled studies indicate that lithium is superior to placebo (Bunney, 8; Goodwin, 9; Maggs, 10; Schou, 11).

The first study was reported in 1954 by Schou et al (11) in which 38 manic patients were treated; this group was subdivided into 30 "typical" and 8 "atypical" cases. Assignment to treatment schedule is reported as follows: "In some cases the lithium was given in an 'open' treatment for a certain period. In other cases, a 'blind' scheme was adopted; the patients receiving lithium salts or placebo for a short period, usually 2 weeks." Medication was shifted every two weeks from lithium to placebo or vice versa. Three lithium salts, the chloride, carbonate, and citrate were used. Clinical response was made on a three-point scale as positive, possible, or negative. Dosage was 24–48 mEq of lithium per day. Of the 30 "typical cases," positive effect was seen in 12, possible effect in 15, and negative effect in 3; of 8 "atypical cases," positive effect was seen in 2, possible effect in 3, and negative effect in 3. The placebo data are presented in a somewhat different form which does not allow for a comparable analysis.

Another controlled evaluation of lithium against placebo was carried out by Maggs (10). Twenty-eight manic patients were randomly assigned to lithium and placebo. One group was treated with a 14-day sequence of lithium–rest–placebo, while the other received placebo–rest–lithium. A fixed dose of lithium carbonate, 1.5 g per day was used, and psychiatric assessments were made using the Wittenborn Scale. Because of toxicity, uncooperativeness, or unmanageability, only 18 of 28 patients completed the entire study. The exclusion of these dropouts may present a methodological problem in the analysis of the results. However, the statistical assessment of the findings on Cluster III (Manic states) and Cluster V (Schizophreniform Excitement) of the Wittenborn Scale showed that the degree of mania diminished significantly more during the second week of treatment with lithium than after similar treatment with placebo.

In studies by Bunney et al (8) and Goodwin et al (9), placebo was substituted for lithium at predesignated periods in a blind fashion. In the former, two manic patients were observed and both were normalized by lithium and both relapsed on its withdrawal. In 10 out of 10 trials, manic behavior increased within 24 hours following substitution of placebo. In the latter, the study was extended to include 12 manics. Nine of the 12 manics were rated as "unequivocal responders" and the remaining 3 failed to improve.

The comparison studies with chlorpromazine are of greater practical importance in the assessment of the therapeutic utility of lithium in this disorder and they will be considered individually.

In 1968, Johnson et al (12) published the first controlled study comparing lithium with another psychotropic agent, chlorpromazine. As a parallel phase of this work a control group of schizophrenic patients with psychomotor excitation and elation were included to assess the effect on hyperactivity in nonmanic excitation. In the former part of the report, 28 manic patients were evaluated. The results of this study indicate a superior therapeutic effect of lithium carbonate in manic states. The global remission rates obtained with lithium were 78 percent as compared with 36 percent of those patients treated with chlorpromazine. It was clear that chlorpromazine produced a decrease in motor activity more rapidly and improved manageability early. Similar improvement with lithium did not occur until an average of 8 days after commencement of treatment had passed. In the nonmanic states a striking dissimilarity of action was noted. The chlorpromazine-treated schizo-affective patients all showed varying degrees of improvement, with 80 percent attaining marked or moderate levels of improvement, whereas only 7 of 14 patients treated with lithium showed moderate improvement. The remainder manifested varying degrees of worsening of their condition. This outcome may be attributed in part to several disputable issues in the experimental design. The dosage plan called for an attempt at matching the approximate number of capsules given per day for each medication and dosage was increased until clinical improvement or toxicity appeared. This may have given rise to the use of too high doses of lithium with high degree of toxicity and aggravation of pathology in schizophrenics and, on the other hand, the maximum dose of chlorpromazine (1800 mg) may be too low.

Spring et al (13) were not able to show a statistically significant difference between the two drugs. In this study, a total of 12 patients were studied (7 on lithium, 5 on chlorpromazine) for a three-week period. Lithium carbonate was given up to 1800 mg/day and chlorpromazine to 1600 mg/day. Then, after the initial 3-week period, if it was felt by the treating psychiatrist that a complete or nearly complete remission had not been achieved, the patients were crossed over to the other drug for an additional 3 weeks. On this procedure, of the 12 patients completing the first 3 weeks of this study, 6 of the 7 patients on lithium responded. Three of the 5 patients on chlorpromazine responded. The 2 chlorpromazine failures were crossed over to lithium and had a complete remission. The lithium carbonate failure was crossed over to chlorpromazine and failed to respond. Also, in comparing the percentage improvements on the eight items of their rating scale, lithium showed a superiority over chlorpromazine on 7 of the 8 items. The conclusions to be drawn from this study must be limited. On percentage outcome, the lithium responders show an 86 percent rate and chlorpromazine a 65 percent rate. However, the small number of subjects studied do not even permit a Chi square analysis to be attempted and therefore no meaningful statistical statement can be made.

In the report by Platman (14), 13 manic patients were treated with lithium carbonate and 10 with chlorpromazine in a double-blind randomly selected drug trial. The author concludes that at the third week of receiving the test drugs, lithium carbonate was superior on all scales of the Psychiatric Evaluation Form (PEF), (Spitzer et al, 15), but this was not statistically significant on any individual parameter. He further states that the general state of the patients on lithium carbonate was markedly superior and, in fact, the majority of the patients were discharged on this drug, while none of the patients on chlorpromazine was sent home. This result is closely akin to the report by Johnson et al (12).

Two recent reports from our unit are also concerned with this question. Johnson et al (16) studied 13 manics on lithium and 8 on chlorpromazine, 7 schizo-affectives, excited phase, treated with lithium and 6 on chlorpromazine, utilizing an array of behavioral rating instruments which included the Treatment Response Assessment Method (TRAM), Brief Psychiatric Rating Scale (BPRS), Clinical Global Impression (CGI), and the Structured Clinical Interview (SCI). On the Tram scale only the lithium-treated manic depressives were significantly improved at termination. The other treatment groups demonstrated no significant change on this scale. On the CGI, lithium and chlorpromazine had a statistically significant effect on all the groups except for the schizo-affectives treated with lithium. On the BPRS, the manic group treated with lithium showed significant changes in more areas of psychopathology such as conceptual disorganization, tension, mannerisms, grandiosity, hostility, uncooperativeness, and excitement. The lack of significant change with chlorpromazine in those items concerned with ideation is in agreement with clinical impressions. Analysis of the SCI scores were carried out by using Penrose's (17) technique. A comparison of the four sets of data reveals that the greatest improvement, over one standard deviation, is shown by the manic patients treated with lithium.

The other study by Shopsin et al (18) underscores lithium's specificity of action. This was a double blind control study of lithium versus chlorpromazine in acute schizophrenic patients. It demonstrated a clear distinction between the effects of these agents and indicated that lithium appears to be without any sedative or neuroleptic properties and, in fact, can precipitate or contribute to further decompensation of schizophrenic symptomatology.

The assessment of these studies allows a conclusion that lithium is effective in the treatment of manic states. However, the question of its comparative efficacy with other standard forms of treatment such as chlorpromazine is not clearly resolved. One limiting factor in these studies is the fact that each had too few cases to allow for adequate statistical analyses. Therefore, the National Institute of Mental Health and the Veterans Administration have organized a multi-hospital collaborative project on lithium therapy to compare its efficacy with chlorpromazine (Prien et al, 19). Twelve VA hospitals and six public or private hospitals participated from which 255 manic patients were admitted into the study and evaluated on the BPRS, IMPS, and Psychotic Inpatient Profile (PIP) rating scales. The mean dosage of the medications was 1800 mg lithium carbonate and 1000 mg chlorpromazine. They were divided into a highly active group of 125 and a mildly active one of 130 cases. In the former group, 38 percent of the lithium patients were dropped as compared to 8 percent of the chlorpromazine patients. In the highly active group comparing lithium completers with chlorpromazine completers (by co-variance analysis), there was no major difference between the groups. A similar conclusion was reached in the mildly active group. Looking at the total group of highly active patients (including dropouts) chlorpromazine was clearly superior to lithium.

This study certainly provides adequate numbers of cases for study and, in fact, is the largest controlled study in the world literature on the chemotherapy of mania. This report goes further in stating that there was no evidence that the treatments acted differentially on the underlying manic process. The latter point is at variance with clinical impressions presented in many uncontrolled studies. The effect of these findings are of immense importance on the practical usefulness of lithium in mania. None of the studies is really comparable from almost every point of view. This latter one also differed in several respects: (*a*) no history of cyclic episodes in the previous two years was requisite for inclusion in the study; (*b*) it was carried out in a regular hospital setting in most cases and not on a research facility; and (*c*) no minimum plasma lithium level was demanded as an inclusion prerequisite. However, some of these considerations may make this study more akin to the procedures adopted in clinical practice. The assessment of these controlled studies does not permit any direct comparisons. Perhaps the single most important issue which makes comparison difficult is the problem of diagnostic specificity and the problems of sample homogeneity.

Thus, even after twenty years of study with lithium carbonate the conclusions that can be reached on its efficacy in acute mania are not absolutely resolved. It is probably safe to say that lithium is an effective agent in the treatment of mania and is superior in efficacy to placebo. With regard to its comparison with chlorpromazine, the smaller control studies suggest its equivalence or superiority to chlorpromazine, whereas the largest study (Prien et al, 19) indicates a superiority of chlorpromazine over lithium.

A number of major differences have been claimed to differentiate the effects of lithium and those of the major tranquilizers used in manic states and they may contribute to an understanding of some of the differences reported above. The major tranquilizers clearly manifest a general central nervous system depressant effect with drowsiness. Thus, a rapid effect is seen in a partial reduction of any psychomotor overactivity. Usually during the first week on these agents, the underlying grandiosity and manic ideation persists, whereas lithium exhibits no such sedative effects. Furthermore, lithium's time course is completely different from the phenothiazines and butyrophenones. Lithium treatment for all practical purposes produces no therapeutic effects for 5 to 8 days after its inception.

PROPHYLACTIC ACTIVITY

The above material demonstrates that it has been difficult to establish the efficacy of lithium in a current manifest manic episode and the task in establishing the prophylactic efficacy of lithium in recurrent mania has been considerably more difficult. Furthermore, no controlled studies have been carried out on the prophylactic activity of any other form of chemotherapy in recurrent mania.

Prophylaxis in recurrent depression has been explored with the tricyclic antidepressant, imipramine, compared with placebo. However, no significant difference in the incidence of recurrent depressive relapses was determined. On attempting to extract material on the prophylactic effects of lithium in manic depressive disease, a claim for such activity emerges from the earlier longitudinal studies. Such references appear in the reports of Noack & Trautner (20) and in reports of Schou et al (11), which suggest primarily activity in the manic phase, and for prophylaxis of recurrent mania only. In a report of Gershon & Yuwiler (21), a similar report of prophylactic activity on recurrent mania is presented. Then, in 1963, Hartigan (22) concluded that a prophylactic action of lithium against recurrent mania occurs and added a claim for its prophylaxis against recurrent depressive cycles. It is important to note that in this report the author adds the reservation that "atypical" cases, in which there was diagnostic doubt because of conspicuous schizophrenic or paranoid features did not do as well as the pure classical forms of mania. Melia (23), in an open trial of lithium in recurrent affective disorders, also concludes that a beneficial effect is produced. His report states that the number of weeks of freedom from relapse for patients receiving lithium was significantly greater at the 0.05 level than the number of weeks of freedom from relapse before lithium. He further states that there was a diminution in the intensity of the mood swings rather than cessation and minor mood swings were indeed manifest, but when on lithium the patients did not require rehospitalization. This latter point is of considerable significance in discussing what in fact is meant by the use of the term "prophylaxis" and will be discussed more fully later in this section. Subsequently two major longitudinal studies on lithium carbonate therapy to evaluate its prophylactic efficacy were carried out. Baastrup (24) and Schou (25) studied 88 patients who had two or more manic depressive episodes during one year, or one or more episodes during two years. Patients were observed for one to six years without lithium and for one to five years with lithium. Angst et al (26), using similar selection criteria, studied 244 patients at three clinics for periods ranging from one and one-half to four years. In both studies, relapse frequency was significantly reduced during lithium treatment. This was true for both bipolar manic depressives and recurrent depressives with no history of mania. The authors concluded that lithium carbonate was highly effective in preventing manic and depressive relapse in recurrent illness. A number of uncontrolled studies followed and these have generally supported the original claim, namely, that lithium serves to lessen the intensity and/or duration and/or frequency of both manic and depressive phase of recurrent affective disorder, whether it be of a unipolar or bipolar nature. Following the first report by Baastrup & Schou (24) there was an intensive critical discussion in British journals, led mainly by Blackwell & Shepherd (27). The points raised include the standard criticisms regarding observer bias and suggestion, together with criticism of the diagnostic criteria for the selection of patients for prophylaxis in the Danish studies. Objection was also taken to Schou's use of the null hypothesis and his experimental design and that neither a control medication nor a placebo group was used. Another point raised was that periods with lithium always followed periods without lithium, so that at the time of lithium treatment patients were older and had been ill for a longer time. However, in contrast to the favorable reports discussed above, Spring et al (28) presented a negative report on the prophylactic activity of lithium carbonate. These investigators treated 9 manic patients with lithium after an acute manic attack, kept them on prophylactic lithium, and followed them for three months to one year. Of the nine patients, five relapsed within one year. In four of the five relapsing patients, the recurrent manic attacks were as severe as any previously experienced. Of the four nonrelapsing patients, the follow-up period was less than their average interval without attacks. Finally, in a longitudinal study by Fieve et al (29), forty-three bipolar manic-depressive patients were followed for periods of five months to over two years in an outpatient clinic. This report indicated that lithium did produce a mild decrease in depth of depression scores among the patients who had been on the drug for over seven months, as compared with patients who had been on lithium for less than seven months. However, there was no significant change in the frequency of depressive failures as determined by test scores and interview ratings. To resolve some of the issues raised by longitudinal trials, Baastrup et al (25) initiated a double-blind comparison of lithium with a placebo utilizing a discontinuation design. The study group consisted of 84 females who had been on maintenance lithium therapy for at least one year. There were two diagnostic groups: (*a*) manic depressives ($N=50$) with a history of both manic and depressive episodes, and (*b*) recurrent endogenous depressives ($N=34$) with no history of mania. Patients were paired within each diagnostic group. One member of each pair was randomly taken off lithium and given identically appearing placebos. Each patient was evaluated periodically by clinicians who were unaware of the patient's treatment assignment. The patient was considered relapsed if he had a manic or depressive episode severe enough to require hospitalization or supplementary drug therapy. The results indicated that in both diagnostic groups lithium was significantly superior to placebo after five months; 55 percent of the manic depressives on placebo had relapsed as compared to none of the patients on lithium. In the recurrent depression group, relapse recurred in 53 percent of the placebo patients and none of the lithium patients. In this study the differences in relapse frequency between periods without lithium and with lithium were statistically significant at the $p<0.001$ level. This study is perhaps the most important and well controlled, and even though not without criticism in regard to methodology, still provides us with a study plan which is acceptable in most respects. It provides the first clear-cut evidence for the prophylactic efficacy of any psychotropic agent. Furthermore, it presents the view that lithium has a prophylactic effect in both manic depressive and recurrent depressive relapses. One important issue, however, is the fact that this was a carefully selected sample of female lithium responders who had been maintained in the program with positive therapeutic effect for at least a year. It is possible that the prophylactic effect of lithium may be less pronounced in a randomly selected group of manics and recurrent depressives.

The fact that this latter point may be significant appears from other studies where a more random selection of cases was utilized. For example, Platman (30) compared lithium with imipramine in 70 patients with recurrent affective disorder. He found that neither lithium nor imipramine demonstrated a major prophylactic effect. Van der Velde (31), in 1970, reported on 75 patients maintained on lithium carbonate treatment. Here, again, the prophylactic efficacy was not as marked as reported in the Danish study. Thus, the situation in regard to prophylaxis has reached an important but critical point where the studies of Baastrup et al (25) and Angst et al (26) provide considerable evidence for the claim of prophylactic efficacy of lithium, and some other studies using different criteria for selection still provide evidence but not as clear-cut as that of the previous authors. In any case, it is important that the effect observed must be more carefully defined. It might be stated that relatively large doses of lithium are required to resolve the acute manic episode and then for maintenance, the patient is continued on lower doses of lithium; in these cases it appears that the prophylactic activity, when present, modifies the magnitude of the amplitude of the manic or the depressive phase rather than abolishing absolutely the endogenous episode. For example, it was noted that when a manic episode begins to appear the patient experiences some of the same features that heralded previous episodes. These may include mild restlessness, insomnia, and overactivity or some more individually specific alteration in behavior. This has been described as hypomanic alert by Jacobsen (32). At such times the dosage of lithium needs to be increased to produce an abatement of symptoms. Thus, the maintenance medication produces a marked diminution in the amplitude of symptomatology and enables the patient and his family to detect and appreciate the onset of the cyclic episode, but the patient then can usually be maintained as an outpatient without requiring readmission to a hospital. Details on treatment procedure are presented elsewhere (Gershon, 4).

LITHIUM IN DEPRESSION

Assuming that lithium is effective in mania, it would seem, at least superficially, that it might be unlikely to be therapeutic in depression. This conclusion seemed to be supported by the earlier workers' reports that lithium was without beneficial effect in a current manifest depressive episode, (Cade, 3; Noack & Trautner, 20; Schou, 6). Subsequently, several authors reported improvement of depression as a result of lithium treatment. Andreani et al (33), stated that 10 of 24 cases improved, and Vojtechovsky (34) reported improvement in 8 of 14 depressed patients who had previously been resistant to ECT. Dyson & Mendels (35) recently reported the outcome of an uncontrolled study of lithium in a mixed group of depressed patients. Of the 31 patients studied, they reported no success in 9 cases of neurotic depression. However, they noted that most of the 19 cases of manic depressive illness, depressed phase, responded to lithium within one week. Goodwin et al (9) reported a double blind placebo study of 8 hospitalized depressed patients and concluded that 2 showed an unequivocal and 3, a probable response to lithium. Fieve, Platman & Plutchik (36) studied 29 endogenous depressed patients and at the end of the three-week treatment period the lithium patients showed slight improvement in their depressions, while the imipramine patients showed moderate-to-marked improvement on the basis of self-rating and objective rating.

At this time, no clear picture has emerged, but more than a suspicion exists that lithium may also exert a therapeutic role in the treatment of a current manifest depression of the endogenous type.

TOXICITY

The acute effects of excessive dosage are well known (Gershon, 4). However, the toxic effects seen with long-term usage have only more recently been described. These latter effects may be of considerable interest to internists and may also throw some light on the mode of action of the lithium ion in affective disease.

The side effects appearing with chronic ingestion of lithium include diabetes insipidus-like syndrome, elevation of blood sugar, goiter, and elevated white blood cell counts.

The occurrence of goiter in patients on lithium treatment was first observed by Schou (37). In reviewing 330 patients on maintenance therapy from 5 months to 2 years, 12 had developed diffuse, nontender thyroid enlargements while remaining clinically euthyroid. Abnormal iodine metabolism was revealed in several patients as indicated by increased tracer uptake and thyroid iodine clearance. The goiters usually disappear when lithium is discontinued or thyroid hormone is administered concurrently with lithium medication. Hypothyroidism with enlargement of the thyroid has also been reported (Shopsin, Blum & Gershon, 38; Wiggers, 39). In the former case, lithium was clearly implicated as causal by a series of withdrawal-reinstitution trials. Sedvall et al (40) reported that serum protein-bound iodine was significantly reduced in both man and rat after one to two weeks of daily lithium administration and I^{131} uptake by the thyroid gland was markedly increased in lithium medicated manic-depressive patients. Possible mechanisms of action have been considered in a review by Shopsin (41).

Another rare effect seen on continued medication is a diabetes insipidus-like syndrome (Angrist et al, 42). This report deals in detail with two cases in which this response occurred while receiving therapeutic doses of lithium with blood levels generally considered to be nontoxic and in whom no other effects of lithium toxicity appeared. The picture was characterized by excessive thirst, high urine volumes, low urine specific gravity, and inability to concentrate urine even under conditions of water deprivation. Both failed to concentrate urine in response to either hypertonic saline or pitressin administration. The condition returned to normal in both cases within three weeks of cessation of lithium. Again, the mechanism was not clearly resolved.

Carbohydrate metabolism has also been implicated as being modified by lithium ingestion. Van der Velde & Gordon (43) reported increased glucose tolerance (GT) in 8 of 10 patients receiving lithium and a decrease following withdrawal of medication. The results of a more recent study by Henninger & Mueller (44) are directly at odds with the former authors. The intravenous glucose test (GTT) and insulin tolerance test (ITT) were employed by Henninger & Mueller (44) in manic patients before and after at least two weeks of lithium carbonate treatment. The results indicated that lithium treatment was accompanied by increased blood glucose levels as well as a decreased sensitivity to insulin. Recent studies carried out by Shopsin et al (45) indicate that increased blood glucose levels resulted after lithium administration in different diagnostic categories and reached statistical significance at the 60-minute interval of the GTT. This report also shows that a single lithium dose given to patients of different diagnostic types significantly increased blood sugar values one half hour after oral administration of glucose with mildly elevated levels persisting 2 hours later compared to baseline assessments. The implications from these studies are that decreased glucose tolerance accompanying lithium administration is due to a physiological effect of this ion and is not related to either psychiatric diagnosis or change in clinical state or duration of treatment with this drug. Again, the mechanism responsible for this action of lithium has not been resolved. A "consistent and striking" elevation in white blood cell (WBC) count accompanying lithium administration was first reported by Mayfield & Brown (46). Three subsequent reports (Johnson et al, 47; Shopsin et al, 48; O'Connell, 49) have also reported similar findings. Significant leukocytosis occurred during periods of lithium ingestion and this phenomenon was reversible, apparently innocuous and not related to psychiatric diagnosis or the many variables of hospitalization. While the elevations in WBC count appear to be due to drug effect, they are not dose-related or dependent on the concentration of lithium found in the peripheral blood. It may be relevant to pursue the explanation for this and some of the other effects mentioned within the framework of lithium effects on endocrine functions. In this regard, Platman & Fieve (50) have found that lithium causes a significant elevation of plasma cortisol levels in the morning. This elevation appeared to be related to increases in plasma lithium levels, and a reduction of plasma cortisol levels occurred on discontinuation of lithium medication.

MODE OF ACTION

Although a considerable amount of work has been undertaken in order to seek the mode of action of the lithium ion in affective disorders, one must conclude that this has not yet been determined. Lithium has been shown to effect electrolytes (Baer, Kassir & Fieve, 51), endocrines (Shopsin, 52), monoamines (Schildkraut et al, 53), and other systems. However, the question still unresolved is which of these effects is primary in regard to its effect on behavior and, furthermore, are any of them central to its effects in manic-depressive disease. For instance, a possible indirect mechanism whereby alterations in electrolyte concentration could change central nervous system function would involve catecholamine metabolism. The concentrations of sodium and potassium have been reported to be important factors in catecholamine metabolism (Bogdanski & Brodie, 54), and lithium-induced changes in brain catecholamine metabolism have been reported (Corrodi et al, 55).

The similarity of lithium to sodium in the periodic table, its ability to substitute for sodium in media surrounding nerves (Hodgkin & Katz, 56), and its active transport in biological systems (Zerahn, 57), present the possibility that a mechanism related at least in part to altered electrolyte metabolism may be relevant in considering lithium's mode of action. It has been shown that lithium produces a natriuresis, kaluresis, and an increase in urine volume in man (Trautner et al, 58; Baer et al, 59). Also certain claims have been presented suggesting that there exists a differential retention and excretion of lithium in the manic phase compared with normals and the normothymic phase of the illness (Trautner et al, 58; Greenspan et al, 60). These observations at least raise the possibility that lithium may alter electrolyte distribution within the body (Mangoni et al, 61; Kerry et al, 62). In certain experimental situations, replacement of sodium by potassium has been demonstrated (Lennon & Lermann, 63) and these experiments raise the possibility that lithium in vivo may also substitute for other cations (Ho et al, 64).

Related to these ionic effects may be altered mineralo-corticoid secretion which may be associated with mental state and which may be modified by the ingestion of lithium. An increase of 17-hydroxycorticosteroids in depressive phase and a decrease of these hormones in the manic phase was observed by Bunney et al (65). Similar changes were reported on plasma 11-hydroxy corticosteroids by Hullin et al (66).

The other major area of biochemical data concerning the affective disorders which has accumulated in recent years deals with catecholamine metabolism and this work has been described as the catecholamine hypothesis by Schildkraut (67). This catecholamine data implies a bipolarity in which depression and mania represent related but opposite states; the former is associated with decreased, and the latter with increased functional norepinephrine at central adrenergic nerve endings. This bipolar theoretical framework cannot easily be invoked with lithium where this agent appears to exert a similar prophylactic effect in both mania and depression (Baastrup et al, 25). Moreover, it is difficult to reconcile this hypothesis with the report that lithium is effective in the treatment of mania and may also be effective in the treatment of a current manifest depressive episode (Goodwin et al, 9; Dyson & Mendels, 35). Perhaps the greatest amount of experimental activity has concerned itself with studies of lithium on catecholamine metabolism. Schildkraut et al (53, 68, 69) investigated the fate of intracisternally injected H^3NE. In rats given 50 mg/kg of lithium, 1, 2, and 3 hours after the injection, they found an increase in the level of H^3 deaminated catechols and a small decrease in H^3 normetanephrine. There was an insignificant increase in H^3O-methylated deaminated metabolites. In a subsequent experiment, Schildkraut (53), on the other hand, reported that there was an insignificant decrease in H^3-normetanephrine and a significant but small decrease in H^3-norepinephrine. Lithium chloride (2.4 mEq/kg) was given i.p. twice daily for a week at two periods just after the injection of H^3-norepinephrine and 150 minutes later. In the former, there was an insignificant decrease in H^3 and endogenous norepinephrine. In the latter time period, there was a decrease in the level of tritiated norepinephrine, normetanephrine, and totally deaminated O-methylated metabolites. Stern et al (70) studied the effect of lithium (3.75 mg/kg) on norepinephrine turnover in rat brain and found that there was almost 95 percent increase in brain norepinephrine turnover without altering the steady- state levels of norepinephrine in the brain. Corrodi et al (55) showed that the administration of lithium to rats in single doses does not change the tissue levels of norepinephrine, dopamine, or serotonin. Sedvall (71) was not able to show any difference in norepinephrine and dopamine turnover in brain. Greenspan et al (72) gave lithium for 10 days in doses up to 3 mEq/kg and found that the lithium more than doubled the efflux of H^3-norepinephrine from the brain. Ho et al (64) gave lithium chloride (2 m Eq/kg) i.p. daily for 28 days to rats. In the lithium-treated rats, the content of 5-HT in the hypothalamus and brain stem showed a significant reduction of 46 percent and 26 percent, respectively, as compared with the controls. Tissue concentrations of both dopamine and norepinephrine showed no significant change in each of the brain areas studied. In the lithium-treated rats, values obtained for the rate of synthesis using whole brain homogenate were 0.37 μg/g/hr compared with the control value of 0.42 μg/g/hr. This decrease in synthesis was not significant. However, in another series of experiments in which the effect was examined on discrete brain areas, there was an increase of 37 percent in the cerebellum, but decreases were found in all other areas with the highest reduction (57.5 percent) in the hypothalamus. The findings of Corrodi et al (73) are in accord with the above data and tend to support the importance of both regional studies and chronic administration to assess such psychotropic drug effects. Sheard & Aghajanian (74) found that 5-HT and 5-HIAA levels were higher in lithium-treated animals than in controls.

It can be seen that the reported effects on amine metabolism differ and thus may be because the experimental conditions in the studies differed widely. Thus, the intensive studies in this area still do not provide an explanation of lithium's mode of action in the treatment of manic-depressive disease.

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PHARMACOGENETICS: DEFECTIVE ENZYMES IN RELATION TO REACTIONS TO DRUGS[1]

7054

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Each time a physician gives a drug to a patient, there is some risk that the therapeutic effects he expects will not be achieved. Adverse reactions and unwanted side effects may occur even though due allowance has been made for the patient's age, sex, weight, disease state, and the other drugs the patient has been receiving. These calculations are usually made upon the assumption that each patient is an "average" patient, and the factors do not allow for individual variability of patients in their response to drugs. It is not surprising to find that some patients do not obtain the desired drug effect, and others show serious adverse reactions when given what is considered to be the standard and safe amount of the drug.

Variation in drug response has been recognized for many years, but it has been only within the past decade or two that genetic differences in people have been studied seriously as a cause for individual variability in drug effects. These pharmacogenetic studies have supplied evidence that there exist wide individual differences in the rate of drug metabolism, the pattern of drug metabolites, and the response to drugs in man. It appears that each person has a particular "pharmacological individuality," like his "biochemical individuality" (1), depending upon his unique genetic constitution. Since genetic variation accounts in large measure for individual variability in drug response, physicians should become familiar with pharmacogenetic principles and the ways in which hereditary traits modify drug effects.

It might be assumed that unusual drug reactions due to inherited traits occur rarely, and only a few therapeutic agents present pharmacogenetic problems. This impression is supported because of the way pharmacogenetics has developed, with emphasis on a relatively few examples characterized by striking clinical symptoms (such as the prolonged muscular paralysis from succinylcholine). The facts are that genetic variability affects the handling and response of most drugs, and we are only beginning to appreciate the importance of these variations in general therapeutics.

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A brief review of drug disposition and metabolism in man makes obvious the many ways by which genetic differences can lead to variable drug effects. Nearly all drugs undergo metabolic transformation during their passage through the liver and other organs. They react with tissue proteins by various processes, such as ionic or covalent binding, they pass through membranes and combine or interact with drug receptor sites. These reactions, particularly those catalyzed by the drug metabolism enzymes, depend upon proteins with a high degree of structural specificity. Genetic mutations that alter the quantity and quality of these proteins can affect drug metabolism, drug binding, and drug-receptor interactions.

Variations in the enzymes of drug metabolism may change two types of metabolic reactions: one, those representing direct structural changes of the drug molecule (oxidations, reductions, hydrolysis), and two, those catalyzed by drug conjugation systems (glucuronidation, sulfation, or acetylation, 2). Inherited deficiencies of these enzymes could retard both metabolism and excretion of a drug and favor its accumulation to toxic concentrations. An enzyme deficiency might also divert the route of drug metabolism to an unusual pathway with the production of unexpected, toxic metabolites. On the other hand, genetic mutations could increase the amount or efficiency of a drug metabolism enzyme. Under these circumstances the usual dose of the drug might never reach effective concentrations in the tissues because of its rapid metabolism.

Genetic mutations can also alter the physiological disposition of drugs. Structural differences in the serum proteins, particularly albumin, could have different binding affinities for drugs and alter the fraction of unbound (free) drug in the blood. Minor changes in binding can modify the concentration of free drug very significantly. For example, at therapeutic concentrations of diphenylhydantoin, about 93 percent of the drug in the blood is bound to plasma proteins (3). Earlier reports (4) indicated that the unbound fraction of diphenylhydantoin varied from 4.1 to 31.4 percent in patients with epilepsy, although Lunde et al (3) found much less individual variation. Unfortunately, few studies of this type have been undertaken, but it is reasonable to suspect that significant hereditary differences do exist in the general population that reduce the binding affinities for certain drugs.

Patients with inherited metabolic disorders, chromosomal defects and other hereditary conditions in which macroscopic or microscopic structural abnormalities are present may also show abnormal or unusual drug reactions. Although the reasons for unusual drug effects in these hereditary conditions are not always understood, the affected patients may require special consideration when given drugs.

Several general reviews, symposia, and conferences on pharmacogenetics have been published during the last few years (5–15). Therefore, only selected examples of inherited differences in drug metabolism, of hereditary resistance to drugs, and inherited disorders in which unusual drug effects have been observed are given in the following sections

INHERITED VARIATIONS IN DRUG METABOLIZING ENZYMES

Individual differences in the rate of drug metabolism (as measured by the fate of disappearance of the drug from the blood) have been recognized for many years. For example, dicumarol (16), antipyrine (17), and phenylbutazone (18) were found to be metabolized in man at rates which vary considerably from individual to individual, but each person duplicated his former metabolism when retested later. This suggested that each person has a characteristic rate of drug metabolism, depending upon his environment (19) and genetic constitution.

One of the first studies calling attention to the importance of genetic factors in dicumarol metabolism was that of Motulsky (20). He found that dicumarol half-life values of siblings correlated closely, as would be required for a genetically determined trait, but the expected correlations between parents and children were not found. He concluded that recessive genes influenced the rate of dicumarol metabolism in some way, but no definite genetic hypothesis could be proposed. Solomon (21) studied a man who metabolized dicumarol very slowly (half-life of 82 hr, versus a normal value of 27 ± 5 hr). The patient's mother may also have been a slow metabolizer of dicumarol. She was unusually sensitive to coumarin anticoagulants and developed a spinal cord hematoma and permanent paraplegia after receiving very small doses of warfarin.

The first study to establish a genetic basis for the metabolism of dicumarol (and also antipyrine and phenylbutazone) was the series of investigations in 14 sets of identical and fraternal twins by Vesell & Page (22–24). Comparison of the half-life values of all three drugs, as determined by the rate of disappearance of the drugs in the blood, showed much less variance between pairs of identical twins than between pairs of fraternal twins. Analysis of the data by the method of Osborne & De George (25) indicated that most of the individual variation observed could be attributed to genetic rather than to environmental factors.

Twin studies to evaluate the contribution of genetics to the variability of drug metabolism in man have also been used by Bönicke & Lisboa for isoniazid (26), Alexanderson et al (27) for nortryptyline, and by Vesell et al (28) for ethanol. From all of these results, it is reasonable to conclude that most of the variation in drug metabolism in man is genetically determined. Twin studies alone, however, do not indicate how many genes are involved, nor how the genes account for the variability. A recent study by Whittaker & Price-Evans (29) indicates that phenylbutazone metabolism is under polygenic control; nortryptyline metabolism also appears to be regulated by several genes (30).

The effects of inducers of drug metabolism, such as phenobarbital (31) and other drugs (27) have also been investigated in twins. Differences in rate of drug metabolism within pairs of identical and fraternal twins were decreased if both received the inducing agent; the differences were increased if one member of the pair were given the inducer. It appears that further studies of this type will be useful in evaluating both genetic and environmental influences, and their complex interactions on drug metabolism.

The aims in pharmacogenetic research are not only to identify new examples of inherited drug reactions and unusual drug effects, but to study the genetic aspects of these conditions. The number of genes involved, their mode of transmission (dominant or recessive; autosomal or sex-linked), the frequency of the genes in the population, the biochemical basis for the unusual drug effects, and the clinical significance of these traits should be established. At the present time, many examples of hereditary differences in drug metabolism are known but our knowledge of most of them is far from complete. A number are listed in Table 1.

The inherited sensitivity to the muscle relaxant drug, succinylcholine, is probably the best understood pharmacogenetic condition (5). The drug is normally hydrolyzed in the serum by cholinesterase, but several variant forms of the enzyme are deficient in this activity.

Two genetic loci have been identified as determinants of serum cholinesterase. One locus, called E_1, is the most important in regulating the quantity and quality of enzyme, and four allelic genes (normal, atypical, flouride and silent) at the E_1 locus account for the normal and variant types of cholinesterase we can identify.

Two mutations produce modified cholinesterases with reduced affinities for choline esters, atypical (or dibucaine-resistant) (32–34), and fluoride-resistant (35); other mutations result in quantitative deficiencies of the esterase so that little if any cholinesterase activity is present in the serum. The heterogeneity of the latter group of mutations is recognized, but collectively they are called silent genes (36–38). The exact nature of the differences in normal (usual) cholinesterase and the atypical and fluoride-resistant variants has not been identified, but the latter may represent single amino acid substitutions in the primary structure. Normal cholinesterase exists in serum in multiple molecular forms (39–41), and the atypical esterase has the same pattern of major and minor components, with essentially the same electrophoretic properties as normal esterase. The modified kinetic characteristics of atypical cholinesterase are expressed in all of its molecular forms (42). Thus, the genetic mutation affects the catalytic site of the enzyme without changing the species of esterase in the blood.

Tests have been developed to classify the type of esterase present in serum (32), and, in some cases, heterozygous carriers of the abnormal genes can be identified. Patients homozygous for the atypical (dibucaine-resistant), fluoride-resistant, or silent genes show prolonged paralysis when given the usual dose of succinylcholine. Artificial respiration may be required for several hours, but with adequate ventilation and care, the patient should recover. The drug is used not only in surgical operations but in electro-shock therapy, and the latter use could be hazardous for those with an abnormal type of esterase, if facilities are not available for proper emergency treatment.

TABLE 1. SOME HEREDITARY VARIATIONS IN HUMAN DRUG METABOLISM ENZYMES OF PHARMACOGENETIC INTEREST

Drug	Enzyme system and variants	Clinical effects	General references
Succinylcholine	Serum cholinesterase		
	Usual or normal	Usual response	(5, 6, 117, 118)
	Dibucaine-resistant (atypical)	Usual response	
	Fluoride-resistant	Prolonged paralysis	
	Silent	Prolonged paralysis	
	Cynthiana	Shortened response	
Isoniazid	Liver N-acetyl transferase		
	Slow acetylators	Cumulative toxicity, peripheral neuritis	(8, 119)
	Rapid acetylators	Expected response	
Diphenylhydantoin	Liver microsomal hydroxylase		
	Slow metabolizers	Cumulative toxicity, nystagmus, ataxia	(71–75)
	Unusual or normal	Expected response	
	Rapid metabolizers	Resistance to drug; more required	
Acetophenetidin	Liver microsomal dealkylase		
	Deficient enzyme	Methemoglobinemia, hemolysis	(76)

Sensitivity to succinylcholine is inherited like a simple, autosomal recessive trait. Heterozygous carriers do not show drug sensitivity (43); they have approximately a 50:50 mixture of variant esterase and normal esterase, and the latter is sufficient protection. About 1:2500 individuals has atypical (dibucaine-resistant) esterase, and 4 percent of the population are heterozygous carriers (44). The other cholinesterase variants are very rare, although the gene frequencies may be somewhat higher in particular ethnic groups or geographic areas. For example, Alaskan Eskimos have an unusually high incidence of people with no detectable serum cholinesterase activity (homozygous for the silent allele) (45).

The Cynthiana variant of serum cholinesterase (Table 1) has been found in only one family (46), but it is an example of a genetic mutation associated with increased, rather than decreased, activity. The individuals have nearly three times as much activity as normal. This appears to be due to an increase in the number of enzyme molecules, rather than a structural change making each molecule a more efficient catalyst (47). People with the Cynthiana variant are resistant to succinylcholine because they hydrolyze the drug rapidly.

Although serum cholinesterase catalyzes the hydrolysis of other drugs containing ester linkages (local anesthetics, 48, aspirin, 49), the clinical significance of cholinesterase variants or esterase deficiency seems to be confined to succinylcholine hydrolysis. Kalow (50) proposed that the action of cholinesterase is important during the time the relaxant agent is undergoing distribution through the blood, but termination of action of the drug, after distribution, depends on diffusion from the neuromuscular junction. This is supported by the failure of concentrated cholinesterase (cholase) to shorten the period of apnea in patients with atypical cholinesterase (51).

Acetylation is an important determinant of the rate of isoniazid metabolism, because the acetylated drug can be excreted by the kidney more efficiently than the free drug. The reaction is catalyzed by an enzyme in the liver, an N-acetyl transferase, and slow acetylators have less enzyme in their livers than rapid acetylators (52, 53).

Approximately half of the population of the United States and Canada are slow acetylators of isoniazid (54). Since the latter are homozygous for a recessive allele, the gene for slow acetylation has an unusually high frequency in the population, 0.72 (55).

During the past decade a number of surveys have been made to determine the proportion of slow acetylators in many different sample populations (56). The range is relatively wide; 5 percent of the Canadian Eskimos (57) to about 83 percent of the Egyptians (58). If slow and rapid acetylators are given the same dosage of isoniazid, the slow acetylators are more likely to accumulate the drug and develop a cumulative toxicity, a peripheral neuritis (59, 60) (Table 1).

Several other drugs, in addition to isoniazid, are acetylated as a major route of metabolism in the body, but it is not certain how many are acetylated by the same N-acetyl transferase. Sulfamethazine acetylation shows the same polymorphism as isoniazid, and this sulfonamide can be used to type individuals as rapid or slow acetylators, just as can isoniazid (61, 69). Hydralazine (61), dapsone (4, 4′-diaminodiphenysulfone) (63, 64) and phenelzine are other drugs believed to be acetylated by the same N-acetyl transferase, but *p*-aminosalicylic acid (52), *p*-aminobenzoic acid (65, 66), and sulfanilamide are acetylated by other N-acetyl transferases. Evans et al (67), in 1965, found that toxic symptoms from phenelzine were more common in slow acetylators, and the lupus erythematosus-like reaction from hydralazine is also more likely to occur in slow acetylators of isoniazid (68).

Individuals can be classified as rapid or slow acetylators by measuring the concentration of isoniazid in the blood 6 hr after giving a standard oral dose of the drug (55). Other methods of classification have been used, such as the half-life of isoniazid in the blood (69) and the rate of acetylation of sulfamethazine (61, 62, 70).

Peripheral neuritis from isoniazid can be prevented by giving the patients extra pyridoxine, and the vitamin does not interfere with the antitubercular activity of the drug (67). Thus, there would seem to be no practical reason for determining a patient's acetylation phenotype. An example (see below) of drug-drug interaction and toxicity demonstrates why it is useful to identify slow acetylators of isoniazid to avoid certain kinds of adverse drug reactions.

Diphenylhydantoin is poorly excreted by the kidney and removal of the drug from the body depends upon its metabolism to a more water-soluble derivative, 5-phenyl-5′p-hydroxyphenylhydantoin, by hydroxylation in the microsomal fraction of liver cells. The hydroxylated derivative is conjugated with glucuronic acid and excreted in the urine.

Kutt et al (71) found a reduced ability to hydroxylate diphenylhydantoin in several members of a family through a patient who developed overdose toxicity symptoms when given the usual doses of the drug. The patient accumulated the drug and excreted much less hydroxylated metabolite in the urine than normal. Two relatives were found to have a similar limited ability to hydroxylate diphenylhydantoin. Presumably, their microsomal hydroxylase is defective, but direct enzymatic tests in vitro have not been made, and another possible explanation for the familial defect should be considered. Diphenylhydantoin, like many other drugs (19), accelerates its own rate of metabolism upon repeated administration. These individuals may have a hereditary deficiency which prevents the usual increase in microsomal enzyme activity (enzyme induction). Whatever the mechanism, the reduced capacity of the affected individuals to metabolize diphenylhydantoin requires that they be given lower doses of the drug than usual to maintain therapeutic tissue concentrations. Kutt has also found a few patients who metabolize diphenylhydantoin much more rapidly than usual, but it is uncertain whether they have inherited a more efficient microsomal hydroxylase or have a greater capacity for enzyme induction with chronic treatment.

Kutt et al (72) found some patients given both diphenylhydantoin and isoniazid in the usual doses developed signs of diphenylhydantoin overdosage (nystagmus, ataxia, and drowsiness). These patients had not shown signs of diphenylhydantoin toxicity previously, and it was obvious that isoniazid had reduced the tolerance to diphenylhydantoin. Kutt et al (73) obtained experimental evidence that isoniazid inhibits diphenylhydantoin metabolism (hydroxylation) in studies with rat liver microsomes in vitro, and it is reasonable to assume that isoniazid acts as an inhibitor of diphenylhydantoin hydroxylation by liver microsomes in these patients.

More recently, Kutt and his colleagues (74, 75) have established that the patients most likely to show intolerance to diphenylhydantoin when receiving both drugs are the slowest acetylators of isoniazid. Thus, an interesting type of drug-drug interaction results from the genetically determined slow acetylation trait, and other examples of this type can be expected to occur.

Shahidi (76) discovered an unusual reaction to acetophenetidin in two sisters in a Swiss family (Table 1). Severe hemolysis and methemoglobinemia developed if either sister were given 2 to 5 g of the drug. At times, the methemoglobin level reached 50 percent and hemolysis was so extensive that blood transfusions were required for the younger sister.

The usual metabolic fate of acetophenetidin in man is 0-dealkylation (0-deethylation) in the liver to N-acetyl-*p*-aminophenol, followed by conjugation to the glucuronide and sulfate derivatives. Shahidi proposed that the two sisters were deficient in the liver microsomal enzyme required to catalyze the 0-dealkylation reaction, and the drug was metabolized by an alternative pathway. The presence of appreciable amounts of 2-hydroxyphenetidin in the urine of these patients suggested that the drug had been deacetylated and then hydroxylated. The resulting *o*-aminophenol metabolite is known to be a toxic compound and to cause hemolysis and methemoglobinemia. The diversion of the drug to another metabolic pathway and toxic metabolite is a reasonable explanation for the unusual clinical symptoms in the sisters. Shahidi (76) found that the administration of phenobarbital for several days followed by a small dose of acetophenetidin to the younger sister resulted in even greater hemolysis and methemoglobin formation. Phenobarbital, a known inducer of the liver microsomal drug metabolism enzymes, did not correct the patient's enzymatic deficiency; in fact, it probably induced the microsomal hydroxylase and increased the production of the toxic metabolite.

INBORN METABOLIC DISORDERS WHICH MODIFY DRUG EFFECTS

Unusual reactions to drugs may be inherited without the hereditary trait being directly associated with the metabolism or disposition of the offending drugs; for example, an inborn error of metabolism may affect the internal environment of cells or tissues so that drugs produce unusual effects in the abnormal environment. For example, a number of inherited enzymatic deficiencies of the red blood cells can be compensated for, under ordinary circumstances (Table 2). These are not drug metabolism enzymes, but they confer unusual susceptibility and adverse reactions if certain drugs are given (9, 77). The best known are the deficiencies of erythrocyte glucose-6-phosphate dehydrogenase (G6PD), and at least 80 distinct variant forms of G6PD have been identified (78). All variants, however, do not cause hemolysis with primaquine and other drugs; Kirkman (79) has pointed out that, in general, the variants with activity less than 30 percent of normal are the ones regularly associated with hemolytic reactions. The exact mechanism of red cell hemolysis is not known (80), but a reduced level of G6PD activity causes these cells to be less efficient in generating NADPH, the reduced cofactor for glutathione reductase. Glutathione is critical for maintaining the integrity of the erythrocyte membrane, and G6PD-deficient cells show a reduction in their glutathione concentration when incubated with acetylphenylhydrazine (81, 82). Drug-sensitive individuals can be recognized by this simple in vitro test. Many drugs and chemicals cause red cell hemolysis

TABLE 2. Hereditary Metabolic and Molecular Disorders of Pharmacogenetic Interest

Inherited condition and Reference	Drugs	Effects
Erythrocyte enzymatic deficiencies		
Glucose-6-phosphate dehydrogenase (78, 79)	Primaquine, Diaminodiphenylsulfone, Sulfonamides, Nitrofuran derivatives	Hemolysis
6-Phosphogluconic dehydrogenase (77)		
Glutathione reductase (9)		
Synthesis of glutathione (9)		
Diaphorase (methemoglobin reductase) (120)	Sulfonamides, Acetanilid, nitrites, Amines	Methemoglobinemia
Hemoglobin variants (13, 84)		
Hemoglobin H (121)	Sulfonamides, nitrites	Hemolysis
Hemoglobin Zurich (122)	Sulfonamides, primaquine	Hemolysis
Liver glucuronyl transferase (5) (Crigler-Najjar syndrome)	Salicylates, menthol, Tetrahydrocortisone	Decreased conjugation as glucuronides
Lesch-Nyhan syndrome (85, 86) (Hypoxanthine-guanine phosphoribosyltransferase deficiency)	Allopurinol, 6-thioguanine 6-mercaptopurine 8-azaguanine azothioprine	Drugs not metabolized to active metabolites
Pentosuria (109)	Aminopyrine, menthol	Increased xylulose excretion

in individuals deficient in G6PD. A selected number of these agents are listed in Table 3. Males are more likely to show the enzymatic deficiency and drug sensitivity than females, since the gene determining the characteristics of G6PD is carried on the X chromosome (83). Some other examples of hereditary metabolic and molecular diseases associated with unusual drug effects are listed in Table 2. Inherited variations in the structure of hemoglobin may predispose a patient to undesirable drug effects, such as methemoglobinemia and hemolysis. Anesthesia is a potential hazard for patients with sickle cell hemoglobin since hypoxia may cause intravascular sickling followed by infarction (84).

Some drugs are administered in an inactive form and must be metab-

TABLE 3. Drugs and Agents That Can Cause Clinically Significant Hemolysis in Glucose-6-PO$_4$ Dehydrogenase-Deficient Individuals

Acetanilid: 3.6 g
Phenylhydrazine: 30 mg
Sulfanilamide: 3.6 g
Sulfacetamide
Sulfapyridine: 4.0 g
Sulfamethoxypyridazine (Kynex): 2.0 g
Salicylazosulfapyridine (Azulfidine): 6.8 g
Thiazolsulfone
Diaminodiphenylsulfone: 200 mg
Trinitrotoluene[a]
Nitrofurazone (Furacin): 1.5 g[a]
Nitrofurantoin (Furadantin); 400 mg
Furazolidone: 400 mg
Furaltodone (Altofur): 1.0 g
Quinidine: 0.8 g[a]
Primaquine: 30 mg
Pamaquine: 30 mg
Pentaquine
Quinocide: 30 mg
Naphthalene
Neosalvarsan: 600 mg
Fava beans

[a] Hemolysis observed principally in Caucasians. From Kirkman (79).

olized to pharmacologically active derivatives by specific enzyme systems in vivo. An inherited deficiency of a drug-activating enzyme would be a pharmacogenetic condition in which drug ineffectiveness is inherited. Patients with the Lesch-Nyhan syndrome and some adult patients with gout have an inherited deficiency of the enzyme, hypoxanthine-guanine phosphoribosyltransferase (H-G-PRT). It has been proposed that they have hyperuricemia because they are unable to convert guanine to guanylic acid and hypoxanthine to inosinic acid. These nucleotides are normally thought to act as "feedback" inhibitors to regulate the rate of purine biosynthesis (85).

In order to act as a feedback inhibitor, allopurinol must be metabolized to its corresponding nucleotide, and this transformation also requires H-G-PRT. As would be expected, allopurinol still inhibits xanthine oxidase in these patients, but it is ineffective in reducing purine biosynthesis. These patients also are unresponsive to other purine antimetabolites which require a conversion to their nucleotides by H-G-PRT to be active. These drugs include 6-thioguanine, 6-mercaptopurine, 8-azoguanine, and azathioprine (86).

HEREDITARY RESISTANCE TO COUMARIN ANTICOAGULANTS

A very unusual type of pharmacogenetic condition in man is the hereditary resistance to the coumarin anticoagulant drugs described by O'Reilly et al (87). They noted a 73-year old man who was given warfarin following a myocardial infarction showed no anticoagulant response to the usual dose of the drug. The dosage was gradually increased and a prothrombin response was finally achieved, but it required 145 mg per day. The mean daily dose of warfarin needed to maintain patients adequately is 6.8 ± 2.8 mg (88), and this patient required approximately 20 times the usual dose. O'Reilly et al

(87) found that the patient did not metabolize warfarin faster than normal, and there were no other unusual differences in the binding of the drug to plasma proteins or in the physiological distribution of warfarin. Other family members were tested and six of them in three generations had a similar resistance to warfarin; one of these was an identical twin of the propositus. O'Reilly & Aggeler (89) concluded from the pedigree that coumarin resistance was inherited as an autosomal Mendelian dominant trait. Resistant individuals were unusually sensitive to the antidotal effects of vitamin K, about 20 times that observed in other patients. O'Reilly et al (88) believe that an enzyme or a receptor site involved in the synthesis of the clotting factors II, VII, IX, and X has been modified by genetic mutation in its affinity for both coumarin anticoagulants and vitamin K (90, 91).

O'Reilly (92) has reported on a second large kindred with coumarin resistance in four generations. Detailed biochemical investigations on the resistant individuals gave very similar results to those from the first kindred.

OTHER HEREDITARY DISORDERS WITH ALTERED DRUG EFFECTS

Certain drugs may produce unusual or undesirable effects in patients with hereditary conditions characterized by gross or microscopic anatomical abnormalities (Table 4). Patients with glaucoma associated with an inherited narrow angle of the anterior chamber (angle-closure glaucoma) may develop increased intraocular pressure from atropine and other mydriatic drugs (5). In addition, glaucoma resulting from an inherited structural difference in the trabecular meshwork system is accentuated by dexamethasone and other corticosteroids (93). Armaly (94) has investigated the possible use of the response to dexamethasone in genetic studies on the inheritance of glaucoma.

Cardiovascular drugs present special problems in patients with idiopathic hypertrophic subaortic stenosis (95). Cardiac failure may become more severe if digitalis is given and the effect could be fatal if the heart size decreases too much, and no longer compensates for the obstruction.

Mongoloid patients are said to be very sensitive to atropine and even the usual preoperative dosage may be fatal. There is little evidence to support this claim, but these individuals show a greater than normal pupillary dilation with local atropine (96–98) and have an increased sensitivity to the drug given systemically (99).

Patients with familial dysautonomia show a marked increase in blood pressure when given norepinephrine (100). The exact nature of the hereditary disorder is not known, but patients with this condition have responses to drugs which would be characteristic of parasympathetic denervation (101). Of interest is the recent finding that their symptoms can be temporarily relieved by treatment with urecholine (102).

Hereditary malignant hyperthemia with muscular rigidity is a rare but dangerous complication associated with anesthesia (103). The etiology of this condition is unknown (104). Frequently, the symptoms followed induc-

TABLE 4. MODIFIED RESPONSE TO DRUGS IN SOME HEREDITARY CONDITIONS

Conditions	Drugs	Effects	References
Porphyria (hepatic)	Barbiturates	Porphyrin synthesis increased	(123–125)
Resistance to coumarin anticoagulants	Warfarin	Effectiveness of drug decreased	(87–92)
Angle closure glaucoma	Atropine	Intraocular pressure increased	(5)
Chronic, simple glaucoma	Dexamethasone	Intraocular pressure increased	(93, 94)
Idiopathic hypertrophic subaortic stenosis	Digitalis	May not increase cardiac output	(95)
Mongolism	Atropine	Response increased	(96–99)
Familial dysautonomia	Norepinephrine	Increased pressor response	(100–102)
	Intradermal histamine	No flare response	
Malignant hyperthermia	General anesthetics	Hyperthermia, ± hyper-rigidity	(103–107)

tion by halothane and succinylcholine, but this combination was not always used. Some patients have malignant hyperthermia and rigidity, others have hyperthemia alone; evidently the syndrome represents more than one disorder (103). Kalow et al (105) have suggested that patients showing muscular rigidity have an abnormality in their intracellular calcium metabolism from studies with skeletal muscle biopsies. Landrace pigs are susceptible to a similar hyperthemia syndrome and recently were found to respond to treatment with procaine (106). These observations in animals could lead to a rational basis of treatment in man (107).

OTHER CONDITIONS OF PHARMACOGENETIC INTEREST

Several hereditary conditions and traits should be mentioned which might be expected to confer some risk or predisposition to unusual drug effects, but evidence for such a relationship is insufficient or negative. Acatalasia (13, 108) and pentosuria (109) are examples of hereditary disorders of this type. Several drugs increase the excretion of xylulose by pentosurics, but this does not seem to be of any clinical significance. Similarly, the atypical alcohol dehydrogenase of liver found by von Wartburg and

Schürch (110) in 20 percent of a Swiss population and 4 percent of an English population could be of importance in the metabolism of ethanol and the higher alcohols. To date, no definite correlations have been noted between the variant enzyme and adverse drug reactions. Genetic traits such as the threshold for tasting phenylthiourea (5) and the ability to smell hydrocyanic acid (5) are interesting genetic markers, but so far, they have not been useful as indicators of adverse drug reactions or drug metabolism variants. In contrast, the higher incidence of thromboembolic complications in young women taking oral contraceptives of blood groups A, B, and AB than in those of group O (111, 112) indicates the advantage of including genetic markers in adverse drug surveillance and epidemiological programs.

There are a number of reports of adverse drug reactions which mention that certain close relatives were similarly affected. Bone marrow aplasia after chloramphenicol in identical twins (113, 114), diffuse myopathy in a mother and daughter following meperidine (115), and jaundice following norethisterone in two sisters (116) are representative examples. Further studies in such families may indicate whether genetic factors contribute significantly to these adverse reactions.

SUMMARY

Considerable progress has been made in recent years in analyzing the various ways genetic traits influence drug response, and how individual genetic differences account for variability in drug effects and drug safety. Pharmacogenetic investigations will continue to identify new genetic markers associated with unusual drug reactions by thorough analysis of affected patients and their relatives. There will also be systematic epidemiological studies to find associations between genetic traits and adverse drug reactions. Both approaches should increase our understanding of man's capabilities and limitations in handling drugs and other foreign chemicals. Therapeutic management, which takes into account each patient's genetic constitution, will improve the usefulness of pharmacological agents and reduce the incidence of undesired and dangerous drug reactions.

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